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PHARMACOLOGICAL   PREVENTION   AND   REVERSION OF ERECTILE DYSFUNCTION AFTER RADICAL PROSTA   TECTOMY, BY MODULATION OF NITRIC OXIDE/cGMP PATHWAYS

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13. SUPPLEMENTARY NOTES

14. ABSTRACT This project aims to find a novel therapy to prevent or correct erectile dysfunction **(ED)** after radi cal prostatectomy **(RP)** for prostate cancer. This was d one by determining in a rat model of bilateral cavernosal nerve resection **(BCNR)**: a) the time course of the histological/biochemical alterations in the penile corpora cavernosa associated with corporal veno-occlusive dysfunction **(CVOD),** the type of ED t hat develops after RP; and b) whether continuous long-term treatment **(CLTT)** with PDE5 inhibitors **(PDE5i)** and/or nitric oxide **(NO)** generators oppose these changes by counteractin g fibrosis, oxidative stress and the loss of smooth muscle cells **(SMC)** subsequent to nerve damage. We have shown that: a) apoptosis, SMC loss, fibrosis, and iNOS induction preceded CVOD; b) CLTT oral PDE5i (tadalafil and sildenafil; as previously vardenafil) at high d oses, prevented these processes; c) oral sildenafil at a lower d ose, + or - the NO generator molsidomine, also prevented CVOD, although the underlying histopathology was less ameliorated than with the higher dose, and the combination treatment did not improve efficacy; d) corporal implantation of mu scle derived stem cells **(MDSC)** prevented CVOD, but th e efficacy was not enhanced by sildenafil. We conclude that CVOD results indirectly from neuropraxia post-RP, and that an early CLTT with PDE5i, as opposed to on demand regimens, prevent CVOD through preservation of the corporal SM. This supports and drives the emerging clinical interest in preventing or "curing" post-RP ED ("penile rehabilitation") with CLTT PDE5i

15. SUBJECT TERMS
Radical prostatectomy, quality of life, erectile dysfunction, PDE5 inhibitors

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INTRODUCTION

This project aims to find a novel therapy to prevent or correct erectile dysfunction **(ED)** after radical prostatectomy **(RP)** for prostate cancer. This was done by determining in a rat model of bilateral cavernosal nerve resection **(BCNR)**: a) the time course of the histological/biochemical alterations in the penile corpora cavernosa associated with corporal veno-occlusive dysfunction **(CVOD)**, the type of ED that develops after RP; and b) whether continuous long-term treatment **(CLTT)** with PDE5 inhibitors **(PDE5i)** and/or nitric oxide **(NO)** generators oppose these changes by counteracting fibrosis, oxidative stress and the loss of smooth muscle cells **(SMC)** subsequent to nerve damage.

BODY

Hypothesis and Specific Aims

Our original **hypothesis** was that ED subsequent to RP for prostate cancer can be prevented and even reversed by long-term sustained treatment with PDE5i, alone or in combination with NO generators, that combat oxidative stress and fibrosis in the penile corpora cavernosa SMC and stimulate cavernosal nerve regeneration, due to the vasculo- and neuroprotective effects of NO and/or its reaction product, cGMP. We aimed to study in a BCNR rat model whether:

1) There is a progressive increase in collagen synthesis and SMC apoptosis in the corpora cavernosa trabecular tissue compounded by a decrease in collagen breakdown, due to production of pro-fibrotic factors such as TGFβ1, that leads to a progressive corporal veno-occlusive dysfunction CVOD**,** the primary form of vasculogenic ED.
2) The induction of inducible nitric oxide synthase **(iNOS)** which is the tissue's endogenous mechanism of defense against fibrosis by its inhibition of collagen synthesis through NO reduction of reactive oxygen species **(ROS)** produced during oxidative stress, can be mimicked by long-term oral treatment with NO generators and/or PDE5 inhibitors, and thus prevent or reverse CVOD, due to the vasculoprotective effects of these agents that increase tissue compliance.
3) These agents lead also to partial penile nerve regeneration, due to their neuroprotective effects.

Our **specific aims** were:

Aim 1. To assess the most effective adjuvant therap y to radical prostatectomy to prevent SM fibrosis and CVOD, b y the modulation of the NO/cGMP/ ROS balance in the peni s subsequent to cavernosal nerve damage in the rat model.

Experiment 1. Time-course of BCNR inducti on of CVOD and the u nderlying penile SM fibrosis.

Experiment 2. Effects of sustai ned oral administration of PDE5 inhibitors or NO generators on CVOD and the underlying corporal fibrosis, at a selected time after BCNR

Rats will be subjected to BCNR or sham-operated, injected in the corpora with a collagen I promoter-β galactosidase construct **(Col I-Pr-Bgal)** and bromodeoxyuridine **(BrdUr),** 7 days and 20 hs before sacrifice, respectively, and sacrificed at 10, 14, 30, 60, 90 days, or only at 90 days after continuous oral treatment with: 1) PDE5 inhibitor (vardenafil), 2) NO donor (molsidomine), or 3) vardenafil/molsidomine combination. **Total: 136 rats.**

Outcomes: a) CVOD, by dynamic infusion cavernosometry **(DIC);** b) fibrosis in corpora and SM by quantitative image analysis **(QIA)** in tissue sections: SM/collagen (Masson), collagen III/I (Sirius red), SMC content (ASMA); c) fibrosis in tissue homogenates: collagen (hydroxyproline), ASMA (western blot).

Aim 2. To optimize the selected treatment to prevent or reverse CVOD after cavernosal nerve damage, and to determine its effect on collagen and SMC turnover, th   e oxidative stress/nitrosative reaction, and nitrergic nerve regeneration.

Experiment 3. Comparison of treatment modalities involving PDE5 inhibitors.
Experiment 4. Time course of BCNR effect   s on collagen and SMC turnov   er rates, oxidative stress, and nitrosative reaction, and modulation of these processes by selected PDE5 inhibitor.
Experiment 5. Effects of selected treatment on nerve regeneration
New rats operated on as in Aim 1 will be also injected 20 h hours prior to sacrifice with BrdUr, and submitted for 90 days to long-term oral treatments with: 1) continuous vardenafil at half the previous dose, 2) as in #1, but with molsidomine (an NO donor); 3) once a day vardenafil; 4) once a day vardenafil plus molsidomine; 5) once a day pentoxifylline, as PDE4 inhibitor; 6) continuous L-arginine, as NOS substrate; 7) selected treatment, but at 45 days post-BCNR for 45 days. In addition, the following 90-day treatment groups will receive also 2 days before sacrifice fluorogold 4% for transneuronal retrograde tracing: 8) BCNR with selected treatment; 9) BCNR untreated; 10) sham untreated. **Total: 85 rats.**
Outcomes: Groups 1-8 will be assayed for: a) as in Aim 1; b) collagen turnover by luminometry & matrix metalloproteinase **(MMP)** activity; c) SMC turnover by QIA for BrdUr and apoptosis; d) Oxidative/nitrosative balance by QIA and western blot for xanthine oxidoreductase **(XOR)**, iNOS, and peroxynitrite. Groups 8-10 will be assayed for: e) regeneration of nitrergic nerves by immunohistochemistry in pelvic ganglion **(IHC)** for PRV/ PnNOS; f) neurofilaments by synaptophysin by IHC/QIA; g) neuron body regeneration by BrdUr-IHC.

Brief rationale

➢ ED is a major risk of RP for prostate cancer and a deterrent for many patients to choose this therapy.
➢ The neuropraxia due to cavernosal nerve damage causes the most prevalent vasculogenic ED: CVOD, or venous leakage. In CVOD blood cannot be efficiently retained in the penis to achieve a rigid erection.
➢ CVOD occurs by the impairment of penile corpora cavernosa relaxation by the loss/damage of SMC (atrophy) and excessive collagen deposition (fibrosis). Cavernosal nerve damage leading to ED is reduced but not eliminated by the use of bilateral nerve sparing surgery, and in any case, practice of this technique is restricted to certain centers of excellence
➢ We showed in rat models that fibrosis of the corpora cavernosa is accompanied by the induction of inducible nitric oxide synthase **(iNOS),** and that gene therapy with iNOS cDNA, or a chronic administration of nitric oxide **(NO)** generators or phosphodiesterase 5 inhibitors **(PDE5i),** such as sildenafil, reduces the corporal fibrosis and SM loss underlying CVOD associated with aging, as well as the penile tunical fibrosis in the localized plaque that characterizes Peyronie's disease.
➢ The main mediator of this endogenously elicited mechanism of defense against fibrosis is the synthesis in the corpora cavernosa of high levels of NO and/or its product, cGMP, maintained during a period long enough as to counteract a chronic process

> ➢ *This SMC protection and antifibrotic effect cannot be achieved by the current clinical administration of PDE5i "on demand",* where the peaks of corporal NO and/or cGMP levels are limited by the pharmacokinetics of each drug, and the duration of sexual stimulation. *The current modality of PDE5i administration is merely palliative and not curative of the corporal histopathology that underlies CVOD.*
> ➢ We showed a similar correction of the corporal histopathology by implanting adult stem cells into the corpora cavernosa of aged rats, reversing the ED associated with aging. We have used skeletal muscle derived stem cells **(MDSC)** and demonstrated that they can originate SMC.

Summary of methodology

A. For published studies supported by this grant

 Surgery and treatments: Nerve resection was performed by resecting the main cavernosal nerves by removing a 3-mm segment uni- or bilaterally. In the sham-operated group, both cavernosal nerves were identified but not resected. Actual drug doses are expressed as mg/kg/day, with the approximate equivalent dose in men as mg/day (bold).
 Animal groups: Five-month-old male Fisher 344 rats were treated and divided as follows:
 Sildenafil at high dose (*n=10/group*): A) sham-operated; B) UCNR, C) UCNR + sildenafil: 20 (200), D) BCNR and E) BCNR + sildenafil: 20 (200). Sildenafil was dissolved in the drinking water and given for 45 days. Washout was 1 day. In a subsequent experiment, treatments for groups D and E were repeated (*n=8/group*), but receiving or not the inhibitor of iNOS activity L-NIL in the water (6.7)
 Tadalafil at high dose *(n=10/group)*: A) sham-operated; B) UCNR, C) UCNR + tadalafil: 5 (50), D) BCNR, and E) BCNR + tadalafil: 5 (50). Tadalafil was dissolved in 10% glucose + 1% Tween 80 and administered retrolingually once per day. Washout was 3 days.
 Time course of events in untreated animals *(n=8/group)*: A) sham operated and B) BCNR groups, left untreated, and sacrificed at 1, 3, 7, 15, 30, and 45 days after surgery.

B. For a manuscript in preparation supported by this grant

 Animal groups. Sildenafil at lower doses, with or without NO donors *(n=8/group)*: 1) Sham; 2) BCNR untreated; 3) BCNR, sildenafil medium dose, in water: 10 (100); 4) BCNR, sildenafil low dose, in water: 2.5 (25); 5) BCNR, sildenafil low dose, retrolingual: 2.5 (25); 6) BCNR, molsidomine IP: 10 (100), 7) BCNR, molsidomine IP 10 (100), with sildenafil medium dose in water 10 (100); and 8) BCNR, molsidomine IP 10 (100), with sildenafil low dose 2.5 (25).
 Sildenafil at very low dose with or without MDSC implantation *(n=8/group)*: 9) sildenafil, very low dose, in water: 1.25 (12.5); 10) sildenafil, very low dose in water: 1.25 (12.5) + MDSC (106 cells), intracorporeal; 11) MDSC (106 cells) alone, intracorporeal.
 Outcome methods for A and B: Dynamic Infusion Cavernosometry (DIC) was applied for measuring CVOD. Quantitative Masson trichrome and immunohistochemical staining in corporal tissue sections for αSMA (alpha smooth muscle actin), apoptosis, proliferating cell nuclear antigen (PCNA), iNOS, and nNOS, was applied, confirming by quantitative western blots. Total collagen was estimated by hydroxyproline assay.

Main Results in relation to planned tasks

Some modifications of the original protocol were introduced, such as replacing vardenafil by sildenafil, bromo-deoxyuridine by proliferating cell nuclear antigen **(PCNA)** and other minor changes Our experimental work investigated different treatment modalities in the BCNR rat model for ED subsequent to RP for prostate cancer, based on the long term sustained administration of PDE5i or of NO donors. This therapy was derived from our previous studies in the rat showing that iNOS is induced during penile corporal and tunical inflammation and fibrosis, thus producing steady high levels of NO and cGMP that instead of inducing SMC apoptosis counteract fibrosis and oxidative stress. PDE5i protect cGMP and NO donors mimic iNOS induction.

We reduced the doses of PDE5i, specifically sildenafil, in the rat model that when translated to men would be equivalent to, or lower than, the ones used for pulmonary hypertension. Subsequently, we decreased further the PDE5i dose, and combined it with an NO donor (molsidomine), to determine whether a similar amelioration of CVOD and of the underlying histopathology could be achieved. This was in order to develop a pharmacological therapy aiming to cure this type of neurogenic/vasculogenic ED with the minimum of side effects, and not just to palliate it, which in fact supported and pioneered, the new approaches for "penile preservation post-radical prostatectomy" that are now being introduced in the clinic. Therefore, our results have considerable translational impact in a topic of high public health significance.

In addition, we have performed studies aimed to determine whether our previous demonstration that muscle derived stem cells **(MDSC)** can correct ED associated with aging (Nolazco G, Kovanecz I, Vernet D, Ferrini M, Gelfand B, Tsao J, Magee T, Rajfer J, Gonzalez-Cadavid NF (2008) Effect of muscle derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. BJU Int, 101:1156-64) can be successfully replicated in the BCNR model, and whether combination with a low dose PDE5i stimulates the MDSC conversion into corporal SMC.

Finally, we have complemented these studies with the parallel ones in another model of erectile dysfunction, the diabetic mouse model lacking inducible nitric oxide synthase (iNOS), where we tested the effects of molsidomine on the underlying histopathology, and we have discussed them in relation to another fibrotic condition of the penis, Peyronie's disease, where long-term sustained administration of PDE5 inhibitors may have therapeutic value.
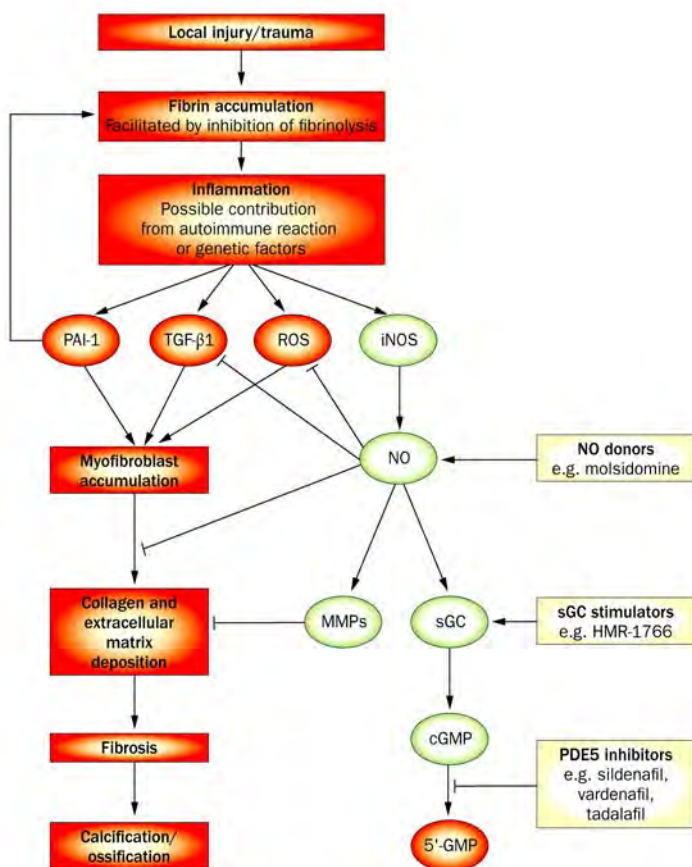

Fig. 1. Proposed mechanisms for the antifibrotic and smooth muscle protective effects of a steady long-term stimulation of the levels of NO and cGMP in the corpora cavernosa. The experimental work described in the subsequent figures has validated our hypothesis but this needs to be tested in clinical trials.
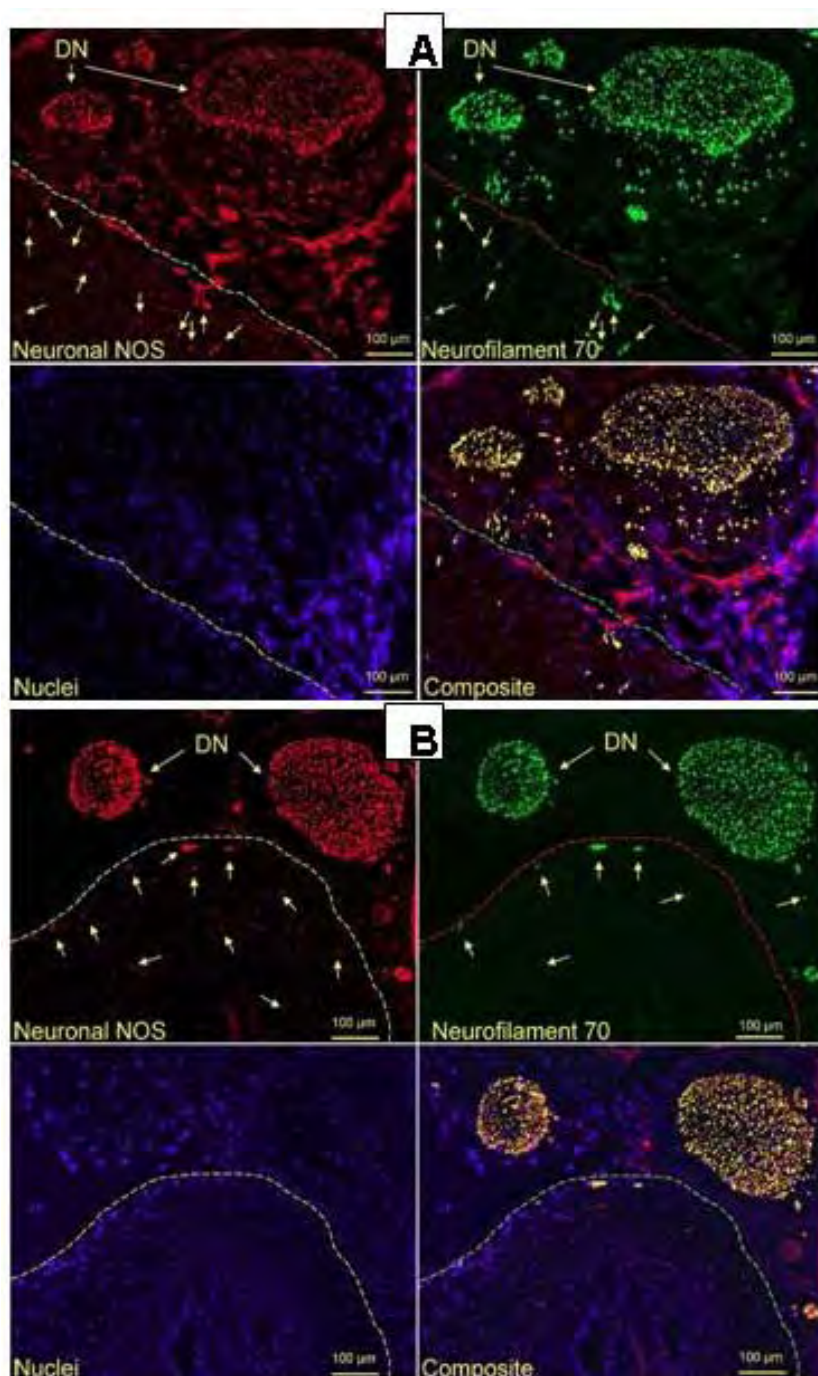
Fig. 2-. **Immunofluorescence analysis of neurofilaments and nitrergic neurofilaments in the penile shaft of rats treated for 3 months with BPA**. Frozen tissue sections were incubated with monoclonal antibodies for nNOS and neurofilament 70 (NF70) and then with either Texas red- or FITC- conjugated seconday complexes. DAPI was used for nuclei (blue). Composite: merge of 3 panels. <u>A</u>: untreated; <u>B</u>: BPA-treated. <u>DN:</u> dorsal nerve. <u>Broken line</u>: outer region of the tunica albuginea. Yellow arrows indicate the presence of nerve terminals in the corpora.

We virtually completed Tasks (Experiments) 1-4 as described under Specific Aims. Task 5 (Effects of selected treatment on nerve regeneration) has not yet been finalized Therefore the two aims have been completed, except for an ongoing experiment that will be reported under paper A-11, to be submitted in June, and presentations B-11 and B-14, to be delivered in May and June. They are based on the detection by double immunofluorescence of the expression of neurofilament 70 **(NF70)** and neuronal nitric oxide synthase **(nNOS)** as markers of nerve terminals and nitrergic terminals, respectively, in the penile sections from the sildenafil, MDSC, and MDSC + sildenafil treated and control rats described in the grant.

Fig. 2 is taken from another project but shows our proficiency in this approach on penile corporal sections from control and treated rats (in this case exposed chronically to bisphenol A, or BPA). nNOS is shown in red and NF-80 in green, with nuclei labed with DAPI (blue) in the control tissue sections **(A),** where the yellow merge indicates the dorsal nerves very rich in nitrergic filaments, and also cavernosal nerve terminals in the corpora. A similar pattern, but with apparently less putative cavernosal nerve terminals is detectable in the BPA specimens **(B).** This analysis when applied to the BCNR specimens will give a precise assessment on the effects of PDE5i and MDSC on nerve regeneration.

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Through this work we have accumulated substantial experimental evidence on the mechanism by which CVOD ensues following RP, and on the putative reversion of these process by sustained production of NO and cGMP through inhibition of PDE5, as outlined on **Fig. 1.** Our results are compiled in a series of reports directly related to the grant, that include 5 experimental papers, 4 review papers/book chapters, and 6 meeting presentations, totaling 60 published or in press figures. In addition, there are some less directly related reports ("supporting" or "derived" results) that include 4 experimental papers and 9 meeting presentations, all listed under Reportable Outcomes. Therefore, we cannot refer here to individual figures other than the two above, but we refer to the directly relevant papers and abstracts that are not reflected in publications by quoting them in the Reportable Outcomes section.

BULLETED LIST OF KEY RESEARCH ACCOMPLISHMENTS

We have demonstrated in a rat model of ED subsequent to cavernosal nerve damage during RP for prostate cancer, elicited experimentally by BCNR, the following main concepts, integrated in the DOD-PCRP 2nd Impact conference **(B-11)**:

Time course study **(A-1):**

➢ Following BCNR, CVOD, measured by dynamic infusion cavernosometry, slowly developed and was detectable at 30 days, and it becoming more pronounced by 45 days.
➢ In contrast, the SMC/collagen ratio in the BCNR corpora was reduced at 7 days and bottomed at 30 and 45 days, due in part to the reduction of SMC, presumably caused by an increase in apoptosis peaking at 3 days. PCNA also peaked at 3 days, but then decayed.
➢ nNOS was reduced early (3–7 days) and disappeared at 30 days, whereas eNOS was not affected. iNOS was induced at day 3, and steadily increased peaking at 30 days.

Effects of CLTT with sildenafil and tadalafil at high doses **(A-2, A-3, A-4-7, A-10, B-11)**

➢ CLTT with sildenafil at a high dose equivalent to 200 mg/day in men, normalized the CVOD, induced by both BCNR and unilateral cavernosal nerve resection **(UCNR).**
➢ The drug prevented the 30% decrease in the penile corporal SMC/collagen ratio, and the 3-4-fold increase in apoptosis and reduction in cell proliferation, and partially counteracted the increase in collagen, seen with both UCNR and BCNR;
➢ The long-term inhibition of iNOS activity exacerbated corporal fibrosis and CVOD in the BCNR rats, but sildenafil functional effects were not affected by L-NIL.
➢ Tadalafil long-term treatment exerted similar effects

Effects of continuous long term treatment with sildenafil at lower doses with or without the NO donor molsidomine, and MDSC with or without sildenafil at very low dose **(A-10, A-11, B-11)**

➢ All treatments (sildenafil alone at a low dose equivalent to 30 mg in men, molsidomine alone, molsidomine with sildenafil, MDSC alone implanted in the corpora, MDSC with sildenafil) normalized the high drop rate during dynamic infusion cavernosometry and improved the response to intracorporeal papaverine.

> The SMC/collagen ratio was significantly increased but not completely normalized by treatment in all series, and sildenafil did not improve the beneficial effects of MDSC

<u>Effects of molsidomine on corporal fibrosis in diabetic mice with genetic blockade of iNOS expression</u> **(A-8, A-9)**

> iNOS genetic deletion, by elimination a steady production of iNOS, NO and cGMP that act as antifibrotic, increased corporal fibrosis and oxidative stress induced by diabetes
> The compensation of iNOS absence by the NO donor molsidomine was effective in reducing local and systemic oxidative stress in the corpora cavernosa, but did not prevent fibrosis.

<u>REPORTABLE OUTCOMES</u>

In yellow highlighting: directly related to the grant proposal and/or to the use of PDE5i and/or molsidomine to prevent or reverse penile fibrosis. The others are on CVOD, and/or penile fibrosis in other contexts.

<u>A. Papers acknowledging this grant PC061300 (W81XWH-07-1-0129) (see Appendix)</u>

1. Ferrini MG, Kovanecz I, Sanchez S, Umeh C, Rajfer J, **Gonzalez-Cadavid NF**. Fibrosis and loss of smooth muscle in the corpora cavernosa precede corporal veno-occlusive dysfunction (CVOD) induced by experimental cavernosal nerve damage in the rat. J Sex Med. 2009 Feb;6(2):415-28. Epub 2008 Dec 2. PMID: 19138364 [PubMed - in process]

2: Kovanecz I, Rambhatla A, Ferrini M, Vernet D, Sanchez S, Rajfer J, **Gonzalez-Cadavid N** (2008) Long-term continuous sildenafil treatment ameliorates corporal veno-occlusive dysfunction (CVOD) induced by cavernosal nerve resection in rats. Int J Impot Res. 2008 Mar-Apr;20(2):202-12.

3. Kovanecz I, Rambhatla A, Ferrini MG, Vernet D, Sanchez S, Rajfer J, **Gonzalez-Cadavid N** (2008) Chronic daily tadalafil prevents the corporal fibrosis and veno-occlusive dysfunction that occurs after cavernosal nerve resection. BJU Int. Jan;101(2):203-10.

4. Rambhatla A, Kovanecz I, Ferrini M, **Gonzalez-Cadavid NF**, Rajfer J. Rationale for phosphodiesterase 5 inhibitor use post-radical prostatectomy: experimental and clinical review. Int J Impot Res. 2008 Jan-Feb;20(1):30-4.

5: **Gonzalez-Cadavid NF**. Mechanisms of penile fibrosis. J Sex Med. 2009 Mar;6 Suppl 3:353-62. PMID: 19267860 [PubMed - in process]

6. **Gonzalez-Cadavid NF**, Rajfer J. Experimental models of Peyronie's disease. Implications for new therapies. J Sex Med. 2009 Feb;6(2):303-13. Epub 2008 Dec 2. PMID: 19138365 [PubMed - in process]

7: **Gonzalez-Cadavid NF**, Rajfer J. Treatment of Peyronie's disease with PDE5 inhibitors: an antifibrotic strategy. Nat Rev Urol. 2010 Apr;7(4):215-21. Epub 2010 Mar 9. PubMed PMID: 20212516.

8. Ferrini MG, Rivera S, Moon J, Vernet D, Rajfer J, **Gonzalez-Cadavid NF**. The genetic inactivation of inducible nitric oxide synthase (iNOS) intensifies fibrosis and oxidative stress in the penile corpora cavernosa in type 1 diabetes. J Sex Med. 2010 Sep;7(9):3033-44.

9. Ferrini MG, Moon J, Rivera S, Rajfer J, **Gonzalez-Cadavid NF**. Amelioration of diabetes-induced fibrosis by antioxidant and anti-TGFβ1 therapies in the penile corpora cavernosa in the absence of iNOS expression. Br J Urol, 2011, in press

10. **Gonzalez-Cadavid NF** (2011) Cellular and molecular basis of new therapeutic approaches for Peyronie's disease and erectile dysfunction (in Spanish), Tratado de Andrologia y Medicina Sexual, Editorial Médica Panamericana., in press

11. Kovanecz I, Rivera S, Nolazco G, Vernet D, Rajfer J, **Gonzalez-Cadavid NF** (2011) Long term daily molsidomine and low dose sildenafil, and corporal implantation of muscle derived stem cells (MDSC), alone or in combination, prevent corporal venooclusive dysfunction (CVOD) in a rat model of cavernosal nerve damage J Sex Med, to be submitted

12. Gelfand R, Vernet D, Kovanecz I, Rajfer J, **Gonzalez-Cadavid NF** (2011) Specific molecular signatures characterize human tunica albuginea fibroblasts, Peyronie's plaque myofibroblasts, and corpora cavernosa smooth muscle cells, and their response to a fibrotic stimulus, To be submitted

13. Kovanecz I, Nolazco G, Ferrini MG, Rivera S, Vernet D, Rajfer J, **Gonzalez-Cadavid NF** (2011) Severe hyperglycemia, renal damage, and low serum testosterone are insufficient in the absence of obesity to cause corporal veno-occlusive dysfunction (CVOD) in a rat model of type 2 diabetes. To be submitted

Note: because of a confusion with the letters PR (which were incorrectly taken as abbreviating the terms "prostate research") there was an unfortunate confusion in the acknowledgement of papers 2 and 3 with another DOD grant: PR064756 (W81XWH-07-01-0181). This error was noticed during the preparation of this report. A request for an Erratum was submitted to the Journal of Sexual Medicine, by substituting grant PC061300 for PR064756, in paper 2, and adding PC061300 to paper 3.

B. Abstracts and presentations related to this grant and to uogenital fibrosis and CVOD

1. Ferrini MG, Kovanecz I, Sanchez S, Rajfer J, **Gonzalez-Cadavid NF** (2007). Time course of corporal veno-occlusive dysfunction (CVOD) development and inducible nitric oxide synthase (iNOS) expression in rat penile tissue after cavernosal nerve damage. Amer Urol Assoc Ann Meet, J Urol 177: 262-62 Suppl. S,

2. Ferrini MG, J Moon J, Rivera S, Rajfer J, **Gonzalez-Cadavid NF** (2009) Genetic Blockade of inducible nitric oxide synthase (iNOS) Intensifies the Development of Fibrosis in a Mouse Model of Diabetes Type 1 Amer Urol Assoc (AUA) Ann Meet, Chicago, IL. Podium Presentation

3. Ferrini MG, Moon J, Rivera S, Vernet D, Rajfer J, **Gonzalez-Cadavid, NF** (2009) Antifibrotic and antioxidant therapies prevent the development of fibrosis in the penile corpora cavernosa associated with type 1 diabetes in the iNOS knock out mouse. Amer Urol Assoc (AUA) Ann Meet, Chicago, IL. Podium Presentation

4. Kovanecz I, Nolazco G, Ferrini MG, Rivera S, Vernet D, Rajfer J, **Gonzalez-Cadavid NF** (2009) Severe hyperglycemia, renal damage, and low serum testosterone are insufficient in the absence of obesity to cause corporal veno-occlusive dysfunction (CVOD) in a rat model of type 2 diabetes. Amer Urol Assoc (AUA) Ann Meet, Chicago, IL. Podium Presentation

5. **Gonzalez-Cadavid NF** (2008) Mechanisms of penile fibrosis. American Urological Association (AUA) Course, 08/20, Baltimore, MD. Invited speaker.

6. **Gonzalez-Cadavid NF**. (2009) Novel insights into the pathophysiology and therapy of erectile dysfunction in type 2 diabetes rat models in the context of reno-vascular disease. NIH Meeting for Urological Complications of Diabetes, 03/11, Baltimore, MD. Invited speaker

7. **Gonzalez-Cadavid NF** (2009) Role of fibrosis and inflammation in diabetes-related urological disorders. Cedars Sinai Meeting on Fibrosis and Inflammation Research, 03/19, 2009, Los Angeles, CA. Invited speaker

8. **Gonzalez-Cadavid NF** (2008) Advances in the understanding of Peyronie's disease at the bench level. Sexual Medicine Society of North America (SMSNA) Ann Meet, 05/18. Orlando, FA. Invited speaker.

9. Vernet D, Heydarkhan S, Kovanecz I, Lue Y-H, Rajfer J, **Gonzalez-Cadavid NF** (2009). Characterization of endogenous stem cells from the mouse penis that express an embryonic stem cell gene and undergo differentiation into several cell lineages. Am Urol Assoc Meet, Chicago, IL, J Urol,

10. Kovanecz I, Rivera S, Nolazco G, Vernet D, Rajfer J, **Gonzalez-Cadavid NF** (2011) Long term daily molsidomine and low dose sildenafil, and corporal implantation of muscle derived stem cells (MDSC), alone or in combination, prevent corporal venooclusive dysfunction (CVOD) in a rat model of cavernosal nerve damage. Am Urol Assoc Annual Meet 14-19 May 2011, Washington, DC

11. **Gonzalez-Cadavid NF,** Kovanecz I, Rivera S, Nolazco G, Vernet D, Rajfer J (2011) Long-term, continuous administration of PDE5 inhibitors alone or combined with implantation of stem cells in the penile corpora cavernosa, prevents erectile dysfunction in a rat model of cavernosal nerve damage after radical prostatectomy. DOD-PCRP 2nd IMPaCT Conference, Orlando, FL, March 9-12, 2011

12. **Gonzalez-Cadavid NF** (2011) Stem cells and iPS as novel potential therapies for erectile dysfunction (2011) Invited speaker. 5th Guandong Society of Andrology conference (Annual Conference 2011) Guangzhou, China, June 18-21

13. **Gonzalez-Cadavid NF** (2011) Experimental approaches to evaluate the epidemiological risk of bisphenol A released from plastics for erectile dysfunction Invited speaker 5th Guandong Society of Andrology conference (Annual Conference 2011) Guangzhou, China. June 18-21 June 18-21

14**. Gonzalez-Cadavid NF** (2011) Long-term continuous treatment of erectile dysfunction with PDE5 inhibitors as a curative antifibrotic approach on the penile corpora cavernosa Invited speaker 5th Guandong Society of Andrology conference (Annual Conference 2011) Guangzhou, China; June 18-21. To be repeated in Beijing.

15. **Gonzalez-Cadavid NF** (2011) Peyronie's disease. A cellular and molecular pathology shared with erectile dysfunction but triggered by different etiologies? Invited speaker Sun Yat-sen University, Department of Urology, Guangzhou, China, June 18-21

C. New applications for funding related to this grant and to uogenital fibrosis and CVOD

The following grant applications have been submitted by investigators in this DOD grant using in part results obtained through this grant.

C1. Funded

1.NIH-NCRR-MBRS Program. PI: Monica Ferrini . Effects of Nitric Oxide and cGMP in a Model of Cavernosal Nerve Damage. 09/09-08/14

2. PI: Rivera, S, Mentor: **Gonzalez-Cadavid NF** (2009) Nitric oxide/cGMP modulationof stem cell differentiation for the treatment of vasculogenic erectile dysfunction caused by cavernosal nerve damage in rodent models. Sexual Medicine Society of North America (SMSNA) research grant. 07/09-06/10.

3. NIH GCRC Medical Student Research Program. PI: **Gonzalez-Cadavid NF** (student: Rivera S (2008) Effects of Nitric Oxide/cGMP Modulators on Fibrosis of the Penile Corpora Cavernosa and Vasculogenic Erectile Dysfunction caused by Cavernosal Nerve Damage in a Rat Model. 11/08-10/09.

4. NIH R21ES019465-01. **PI: Gonzalez-Cadavid)** Bisphenol A effects on the peripheral mechanisms of penile erection 09/10-08/12.

5. NIH R21ES019465-01. PI: **Gonzalez-Cadavid NF** (student: Denesse Segura) NIH/NIEHS Minority Student Supplement to Bisphenol A effects on the peripheral mechanisms of penile erection 05/01/11-08/31/12.

C2. Submitted (Pending)

6 NIH NIDDK 1R21DK089996-01. PI: **Gonzalez-Cadavid NF** Human iPS in erectile dysfunction after radical prostatectomy in rat models. First submission scored 30 and was considered for funding but finally was not awarded. 09/11-08/13

7. NIH NIEHS 1U01ES020887-01. PI: **Gonzalez-Cadavid NF** Cellular-molecular signature and mechanism of BPA effects on penile erection. 10/01/11-9/30/15.

C3. To be submitted by 07/11

8. NIH NIDDK RO1DK53069-07 (resubmission). PI: **Gonzalez-Cadavid NF**. Erectile Dysfunction and Nitric Oxide Synthase in Aging. 11/11-10/15.

9. Department of Defense (DOD) Prostate Cancer Research Program (PCRP) Synergistic Idea Development Award. PI: **Gonzalez-Cadavid NF**. iPS for erectile dysfunction after radical prostatectomy. 12/11-11/14

C4. Not funded

1. Modulation of stem cell differentiation in diabetes-related erectile dysfunction **(PI: Gonzalez-Cadavid NF)**. Submitted on 03/17/08 as main research grant in the O'Brien Urology Center at LABioMed Harbor-UCLA, to NIH-NIDDK in response to RFA. Scored, but not funded

2. Repair of cavernosal nerve damage using stem cells and nitric oxide upregulators (PI: Ferrini, MG)**.** Submitted on 03/17/08 as main research grant in the O'Brien Urology Center at LABioMed Harbor-UCLA, to NIH-NIDDK in response to RFA. Scored, but not funded

3. NIH O'Brien Urology Center on Erectile Dysfunction at LABioMed Harbor-UCLA (Associate Director: **Gonzalez-Cadavid NF)** Submitted 03/17/08 as a grant to fund an Erectile Dysfunction Center based at LABioMed, to NIH-NIDDK in response to RFA. Scored, but not funded

4. NIH Recovery Challenge Grants. PI: **Gonzalez-Cadavid NF**. Modulation of human iPS differentiation in radical prostatectomy-related erectile dysfunction in rat models 09/09-08/11. Scored 35 in the 5 percentile and was considered for funding but finally was not awarded.
.
5. Animal Models of Diabetes Complication Consortium **(PI: Gonzalez-Cadavid)** A diabetes mouse model for studying endogenous/exogenous stem cell interaction. 08/10-07/11.

D. Appointments and distinctions

The personnel paid from this grant included (current positions in parenthesis):

1. Nestor Gonzalez-Cadavid, PhD (Professor), PI
2. Monica Ferrini, PhD (Assistant Professor), Co-I
3. Istvan Kovanecz, PhD (Assistant Professor), Co-I
4. Dolores Vernet, PhD (Research Associate)
4. Gaby Nolazco, MS, (Research Assistant)

In part because of the successful outcomes of this grant during the first year, the principal investigator responsible for the LABioMed site, Dr. Monica G. Ferrini was recruited as full time faculty at Charles Drew University, where she is pursuing her own independent research career. Therefore, she resigned her LABioMed position and her site-PI role in this grant.

A collaborator at LABioMed, Dr. Istvan Kovanecz, was selected to act as new PI at LABioMed, since he is first author in two of the above papers and coauthor in most of them, and is very experienced in animal work and immuno-histochemistry. Concurrently, he was appointed as Assistant Professor in the Research Career Series at the UCLA Department of Urology.

Another collaborator at LABioMed, Steve Rivera, MD, succeeded in his applications for a highly competitive Urology residency, also partly on the basis of his research performance on this grant, and the related ones that paid his stipend as fellow.

The results obtained from this project helped the PI to obtain the following distinctions during 2009-10:

a. 2009-13. Regular member, NIH UKGD Urology Study Section
b. 2010-15. Editor in Chief, International Journal of Impotence Research (2010-15)
c.2010-on. Member of the Editorial Board, Archivos Espanoles de Urologia
d. 2010-on. Permanent member, International Academy of Sexology
e. 2010. Invited speaker, several domestic and international meetings on sexual dysfunction (see CV)

f. 2011. Invited to a press conference organized by the PCRP Impact meeting
g. 2011. Invited speaker to give 4 lectures in China in Guangzhou and Beijing, next June

CONCLUSIONS

➢ Our studies support the concept that vasculogenic ED results from cavernosal neuropraxia during RP, causing the early loss of corporal SMC by apoptosis, which the initial cell replication response cannot counteract, followed by fibrosis. The time course of iNOS induction supports the antifibrotic role of iNOS
➢ An early long-term continuous treatment with PDE5i, even at low doses, as opposed to current on demand regimens, may prevent CVOD through correction of the underlying corporal histopathology.
➢ We also propose that the intracorporeal implantation of stem cells may facilitate corporal tissue repair and prevent CVOD subsequent to RP, but further studies are needed to optimize the cell type, regimen of implantation, and the combination or not with PDE5i
➢ These results provide scientific bases for the emerging clinical interest in preventing or "curing" ED with PDE5i, rather than merely using them palliatively to facilitate an erection and for pharmacological "penile rehabilitation" after RP.
➢ Clinically, our experimental data support the recent approval of tadalafil for daily use, with both 5 and 2.5 mg tablets appearing to be effective in a 14 days treatment (Seftel et al, J Urol 2011, 185:243-48), although longer studies are needed to establish curative effects. In addition, nightly sildenafil (50 mg) for 9 months, one month after RP, enhances penile recovery (McCullough AR, J Urol 2010, 183:2451-6)

REFERENCES

They are listed in the papers above

Fibrosis and Loss of Smooth Muscle in the Corpora Cavernosa Precede Corporal Veno-Occlusive Dysfunction (CVOD) Induced by Experimental Cavernosal Nerve Damage in the Rat

Monica G. Ferrini, PhD,*† Istvan Kovanecz, PhD,†‡ Sandra Sanchez, MSc,‡ Chiome Umeh, B.S,‡ Jacob Rajfer, MD,†‡ and Nestor F. Gonzalez-Cadavid, PhD*†‡

*Charles Drew University-Department of Medicine, Los Angeles, CA, USA; †David Geffen School of Medicine at UCLA-Department of Urology, Los Angeles, CA, USA; ‡Los Angeles Biomedical Research Institute (LABioMed) at Harbor-UCLA Medical Center, Division of Urology, Torrance, CA, USA

ABSTRACT

Introduction. Corporal veno-occlusive dysfunction (CVOD), which usually is associated with a loss of smooth muscle cells (SMC) and an increase in fibrosis within the corpora cavernosa, can be induced by an injury to the cavernosal nerves. The corporal tissue expresses inducible nitric oxide synthase (iNOS), presumably as an antifibrotic and SMC-protective response.

Aims. We studied the temporal relationship in the corpora between the expression of iNOS, other histological and biochemical changes, and the development of CVOD, after bilateral cavernosal nerve resection (BCNR) in the rat.

Methods. Rats underwent either BCNR or sham operation. Cavernosometry was performed 1, 3, 7, 15, 30, and 45 days (N = 8/groups) after surgery. Penile tissue sections were subjected to Masson trichrome staining for SMC and collagen, and immunodetection for alpha smooth muscle actin, iNOS, neuronal NOS (nNOS), endothelial NOS (eNOS), proliferating cell nuclear antigen (PCNA), and terminal transferase dUTP nick end labeling (TUNEL). Quantitative western blot analysis was done in homogenates.

Main Outcome Measures. Time course on the development of fibrosis and CVOD.

Results. Following BCNR, CVOD was detectable 30 days later, and it became more pronounced by 45 days. In contrast, the SMC/collagen ratio in the BCNR corpora was reduced at 7 days and bottomed at 30 and 45 days, due in part to the reduction of SMC, presumably caused by an increase in apoptosis peaking at 3 days. PCNA also peaked at 3 days, but then decayed. nNOS was reduced early (3–7 days) and disappeared at 30 days, whereas eNOS was not affected. iNOS was induced at day 3, and steadily increased peaking at 30 days.

Conclusions. CVOD develops in the BCNR rat as a result of the early loss of corporal SMC by the neuropraxia-induced apoptosis, which the initial cell replication response cannot counteract, followed by fibrosis. The time course of iNOS induction supports the antifibrotic role of iNOS. **Ferrini MG, Kovanecz I, Sanchez S, Umeh C, Rajfer J, and Gonzalez-Cadavid NF. Fibrosis and loss of smooth muscle in the corpora cavernosa precede corporal veno-occlusive dysfunction (CVOD) induced by experimental cavernosal nerve damage in the rat. J Sex Med 2009;6:415–428.**

Key Words. Fibrosis; Erectile Dysfunction; Smooth Muscle; Nerve Sparing; Radical Prostatectomy; Penis; Nitric Oxide; cGMP; Collagen; Inducible Nitric Oxide Synthase; Apoptosis

Introduction

Despite the use of nerve-sparing surgical techniques during radical pelvic surgery in men, the cavernosal nerves still appear to be somewhat susceptible to injury during the surgical procedure as evidenced by persistent and relatively high rates of erectile dysfunction in the immediate post operative period following such nerve sparing techniques [1–4]. The primary reason for this surgically induced impotence is corporal veno-occlusive dysfunction (CVOD) or

venous leakage [5–8] which becomes manifest whenever there is a decrease in the content of corporal smooth muscle cells (SMC) [9]. When this occurs, the remaining corporal smooth muscle mass is unable to achieve sufficient relaxation to attain the high intracorporeal pressures which are necessary for the passive occlusion of the veins that egress the corporal bodies as they traverse underneath and through the tunica albuginea of the penis.

We have previously demonstrated in the rat, in a model of cavernosal nerve resection, that CVOD is apparent at 45 days after the neural injury [10–13]. This functional impairment was associated with a decrease in the SMC mass and an increase in collagen content in the corporal tissue. In addition, we also observed a concomitant increase in the expression of the inducible nitric oxide synthase (iNOS) following bilateral cavernosal nerve resection (BCNR). Since we have shown in other experimental injury models that the upregulation of iNOS postinjury, presumably via the synthesis of NO, can act as an antifibrotic defense mechanism against the development of fibrosis, we then hypothesized that the iNOS may be acting in a similar manner on the corporal tissue in this BCNR model. The evidence to support this hypothesis comes from our finding that the long-term continuous oral administration of a PDE5 inhibitor, which is known to upregulate the action of nitric oxide, not only prevented both the BCNR-induced CVOD and the loss of the corporal SMC mass [10–12] normally seen following this type of injury, but there was the unexpected finding that the PDE5 inhibitors also enhanced replication of the corporal SMC themselves.

However, even though it has been well established that CVOD develops after BCNR and that iNOS expression is increased in the corporal tissue, the temporal relationship between these processes have never been fully elucidated. The aim of this study was to determine: (i) whether the development of the histological and biochemical changes that occur after BCNR precedes the onset of the CVOD; and (ii) when and how long does iNOS induction occur following such a neural injury. These observations would help establish the time frame of when to initiate treatment with PDE5 inhibitors following cavernosal nerve damage in order to achieve the optimum antiapoptotic and antifibrotic effect of these drugs.

Materials and Methods

Animal Treatments

Five month-old male Fisher 344 rats (Harlan Sprague–Dawley, San Diego, CA) were randomly divided into sham operated and BCNR groups. Animals were sacrificed at 1, 3, 7, 15, 30, and 45 days after surgery (n = 8 each group). BCNR was performed as previously described [9–12]. Animals were operated under aseptic conditions and isoflurane anesthesia. In supine position, a midline incision was done, the pelvic cavity was opened, and the bladder and prostate were located. Under an operating microscope, the major pelvic ganglion and its inflow and outflow nerve fibers were identified after removing the fascia and fat on the dorsolateral lobe of the prostate. The main branch of the cavernosal nerve is the largest efferent nerve, which runs along the surface of the prostatic wall. Above the main branch, there are another four to six small efferent fibers which also run toward the membranous urethra, considered as ancillary branches of the CN. In order to recognize the main cavernosal nerve, stimulation with an electrode to induce penile erection was applied. In the sham-operated group, both cavernosal nerves were identified but not resected. In BCNR, the main cavernosal nerves and ancillary branches were resected by removing a 5-mm segment. This procedure mainly eliminates the nitrergic non adrenergic non cholinergic (NANC) stimulation to the corporal smooth muscle that elicits its relaxation during penile erection, while also interrupting some vasoconstrictor neurotransmission through coalescent adrenergic fibers in the cavernosal nerve. All animal experiments were approved by the Institutional Animal Care and Use Committee at our institution.

Dynamic Infusion Cavernosometry (DIC)

Cavernosometry was performed as previously described [10–12,14]. Briefly, basal intracavernosal pressure (ICP) was recorded, and 0.1 mL papaverine (20 mg/mL) was administered through a cannula into the corpora cavernosa. The ICP during tumescence was recorded as "ICP after papaverine." Saline was then infused through another cannula, increasing infusion rate by 0.05 mL/min every 10 seconds, until the ICP reached 80 mm Hg ("maintenance rate"). The "drop rate" was determined by recording the fall in ICP within the next 1 minute after the infusion was stopped.

Histochemistry and Immunohistochemistry

After cavernosometry, animals were sacrificed, and the skin-denuded penile shafts were fixed overnight in 10% buffered formalin, washed, and stored in alcohol (70%) at 4°C until processed for paraffin-embedded tissue sections (5 μm). Adjacent tissue sections were used for: (a) Masson trichrome staining for collagen (blue) and SMC (red); (b) immunodetection with: (i) monoclonal antibodies against α-smooth muscle actin (ASMA) as a SMC marker (Sigma kit, Sigma Diagnostics, St Louis, MO, USA) and proliferating cell nuclear antigen (PCNA) as marker of cell proliferation (Chemicon, Temecula, CA, USA); (ii) polyclonal antibody against iNOS [15] (Calbiochem, La Jolla, CA, USA); (iii) monoclonal antibody against eNOS [16] (Calbiochem); (iv) monoclonal antibody against nNOS [17] (Calbiochem). The specificity of the antibodies was validated by western blot.

Sections were then incubated with biotinylated antimouse IgG (ASMA PCNA, eNOS, nNOS) or biotinylated antirabbit IgG (iNOS), respectively, followed by avidin-biotinylated enzyme complex (Vector Labs, Temecula, CA, USA) and 3,3′diaminobenzidine (Sigma) (PCNA and iNOS), or with the ASMA Sigma kit (ASMA) and 3-amino-9-ethylcarbazole. TUNEL assay was performed as described [10–13] by applying the Apoptag peroxidase detection assay (Chemicon) with TdT enzyme and antidigoxigenin-conjugated peroxidase and 3,3′diaminobenzidine/H2O2. Sections were counterstained with hematoxylin. Negative controls in the immunohistochemical detections were done by replacing the first antibody with IgG isotype. The negative control for TUNEL was by substituting buffer for the TdT enzyme. Testicular tissue sections were used as positive control.

Quantitative Image Analysis (QIA)

QIA was performed by computerized densitometry using the ImagePro 4.01 program (Media Cybernetics, Silver Spring, MD, USA), coupled to an Olympus BHS microscope (Olympus America, Inc., Melville, NY, USA) equipped with an Olympus digital camera [11–15]. For Masson staining, 40× magnification pictures of the penis comprising half of the corpora cavernosa were analyzed for SMC (stained in red) and collagen (stained in blue), and expressed as SMC/collagen ratio. For ASMA and iNOS staining, only the corpora cavernosa were analyzed in a computerized grid and expressed as % of positive area vs. total area of the corpora cavernosa. For PCNA and TUNEL determinations, the number of positive cells at 400× was counted, and results were expressed as a % of positive cells/total cells in the corpora cavernosa. In all cases, two fields at 40× (both sides of the corpora cavernosa) or eight fields at 400×, were analyzed per tissue section, with at least four matched sections per animal and eight animals per group.

Western Blot Analysis

Penile tissue homogenates (100 mg tissue) were obtained in tissue protein extraction reagent (PIERCE, Rockford, IL, USA) and protease inhibitors (3 μM leupeptin, 1 μM pepstatin A, 1 mM phenyl methyl sulfonyl fluoride), and centrifuged at 10,000 g for 5 minutes. Supernatant proteins (30–50 μg) were subjected to western blot analyses [17–20] by 7–10% Tris-HCl polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) in running buffer (Tris/glycine/sodium dodecyl sulfate). Proteins were transferred overnight at 4°C to nitrocellulose membranes in transfer buffer (Tris/glycine/methanol), and the next day, the nonspecific binding was blocked by immersing the membranes into 5% nonfat dried milk, 0.1% (v/v) Tween 20 in phosphate buffered saline (PBS) for 1 hour at room temperature. After several washes with washing buffer (PBS Tween 0.1%), the membranes were incubated with the primary antibodies for 1 hour at room temperature. Monoclonal antibodies were as follows: (i) ASMA, as described above (1/1,000) (Calbiochem); (ii) glyceraldehide-3-phosphate dehydrogenase (GAPDH) (1/10,000) (Chemicon International); and (ii) PCNA (Chemicon International). The washed membranes were incubated for 1 hour at room temperature with 1/3,000 dilution (antimouse), followed by a secondary antibody linked to horseradish peroxidase. After several washes, the immunoreactive bands were visualized using the enhanced chemiluminescence plus western blotting chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA). The densitometric analyses of the bands were performed with Image J (NIH, Bethesda, MD, USA). A positive control was run throughout all gels for each antibody to standardize for variations in exposures and staining intensities. Negative controls were performed omitting the primary antibody. Band intensities were determined by densitometry and corrected by the respective intensities for a housekeeping protein, GAPDH, upon reprobing.

Statistical Analysis

Values were expressed as mean ± standard error of the mean. The normality distribution of the data was established using the Wilk–Shapiro test. Multiple comparisons were analyzed by a two-factor (time and treatment) analysis of variance (two-way ANOVA), followed by post hoc comparisons with the Bonferroni test, according to the GraphPad Prism V 4.1 (GraphPad Software, Inc., La Jolla, CA USA). Differences were considered significant at $P < 0.05$.

Results

Alterations in the SMC/Collagen Ratio in the Corpora Cavernosa Precede the Onset of CVOD Following BCNR

DIC was performed at 1, 3, 7, 15, and 30 days after cavernosal nerve injury in order to determine when CVOD occurs post-BCNR. DIC values for the 45-day time period were taken from one of our previous articles with identical sets of BCNR- and sham-operated rats [11]. However, in all the subsequent figures for histological observations, the representative micrographies for 15 and 45 days are omitted to reduce space. Figure 1 (top) shows that the peak ICP following papaverine injection was not significantly affected by BCNR during the observed 30 days postinjury, although at 45 days after surgery, the value was significantly reduced. The drop rate, however, began to slowly increase by 7 days, but only became significant by 30 days and markedly progressed by 45-day postinjury.

Evaluation of the smooth muscle and collagen content within the corpora was then performed in cross-sections of the penile tissue harvested from the animals following performance of DIC. Figure 2 (top) shows that there does not appear to be any obvious visual changes in the Masson trichrome staining for collagen and SMC on representative micrographs in the sham groups throughout the experiment. However, a progressive intensification of the collagen deposition (stained in blue) and a reduction in the smooth muscle (stained in red) started to be visualized at day 7 after BCNR. When QIA was performed (Figure 2, bottom), an alteration in the SMC/collagen ratio is detected as early as day 3 postinjury, which becomes significantly severe by day 7 and remains so for the remainder of the study. The red staining of the SMC was easily differentiated from the red blood cells, which were not considered in the QIA determinations.
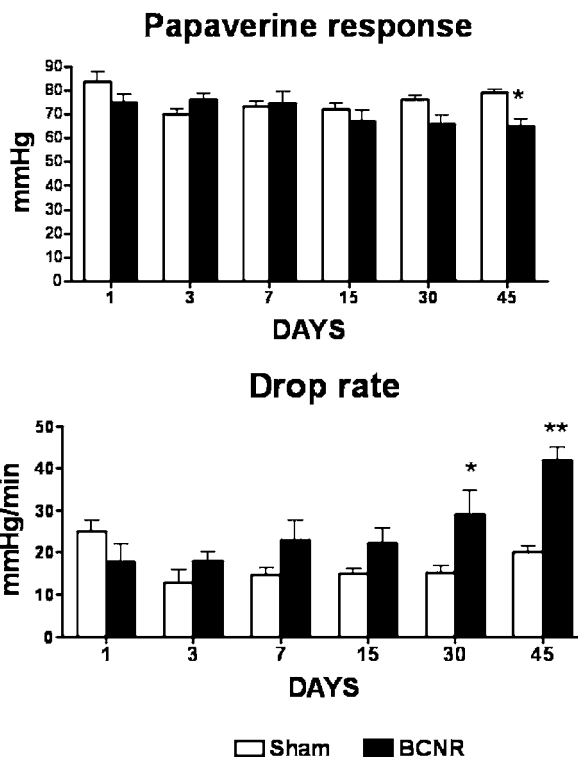

Figure 1 Time course of effect of bilateral cavernosal nerve resection on the erectile function of the rat measured by pharmacological and infusion cavernosometry. Top: Response of the intracavernosal pressure to papaverine; Bottom: Response of the intracavernosal pressure to the interruption of saline infusion. *$P < 0.05$; **$P < 0.01$. SHAM = sham-operated animals; BCNR = animals subjected to nerve resection and killed at 1, 3, 7, 15, 30, and 45 days after surgery.

Picrus sirius red assays and observation under polarized microscope were done in adjacent sections to the ones used for Masson in order to discriminate the collagen III/I ratio. We have found that at the time points 7 and 30, there is an increase in collagen III/I ratio toward more production of collagen III, whereas at 45 days, the ratio is inverted to more collagen I than III (not shown). This difference could be due to the fact that the rate of collagen III synthesis is much faster than collagen I.

A second procedure to estimate SMC content based on the immunohistochemical determination of ASMA, an accepted marker of SMC in the corpora cavernosa, was also performed. Figure 3 (top) shows a considerable reduction with time in ASMA staining in the BCNR group as compared with the sham group as early as 3 days after BCNR that progressively worsens at 30 and 45 days. The respective reductions in
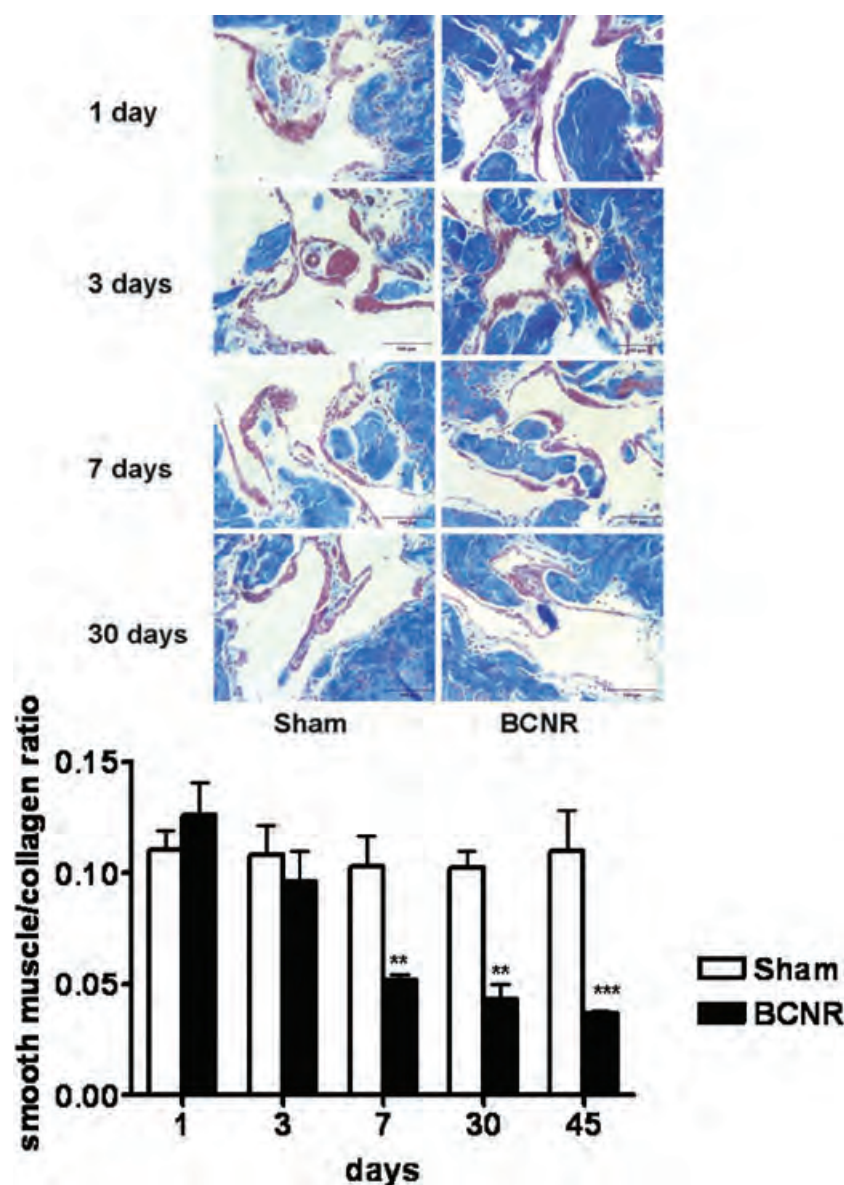
Figure 2 Time course of the effect of bilateral cavernosal nerve resection on the smooth muscle/collagen ratio in the rat corpora cavernosa. Penile corpora cavernosa tissue sections from the rat groups presented on Figure 1 were stained with Masson trichrome. Top: representative pictures (200×, Bar = 100 μm). Bottom: quantitative image analysis. **$P < 0.01$, ***$P < 0.001$. SHAM = sham-operated animals; BCNR = animals subjected to nerve resection and killed at 1, 3, 7, 30, and 45 days after surgery.

ASMA content determined by QIA (Figure 3A, bottom) were 40%, 76%, and 78% at 7, 30, and 45 days, respectively, postinjury. The expression of ASMA in the sham-operated group remained unchanged throughout the experiment (not shown). When western blot analysis of ASMA expression in homogenates of penile shaft tissue (Figure 3B, bottom) was performed, it paralleled the immunohistochemical measurements. Collectively, these results suggest that the histological changes induced by BCNR precede, as expected, the functional impairment of vasculogenic erectile response, and that the earliest event is SMC loss rather than collagen deposition. This is based on the fact that the reduction in

ASMA + cells is rather considerable at a period (3 days) when the SMC/collagen ratio has only slightly decreased.

BCNR Results in a Decrease in nNOS, an Increase in iNOS and No Change in eNOS Content in the Corpora

Since BCNR causes damage to the axons of the cavernosal nerve, Figure 4A confirms by immunohistochemistry with an antibody selective for nNOS a decrease in nNOS staining that is seen in cross-sections of the cavernosal nerve as early as 24 hours following the nerve injury. This antibody does not cross-react with eNOS and iNOS. Because the decrease in staining intensity was
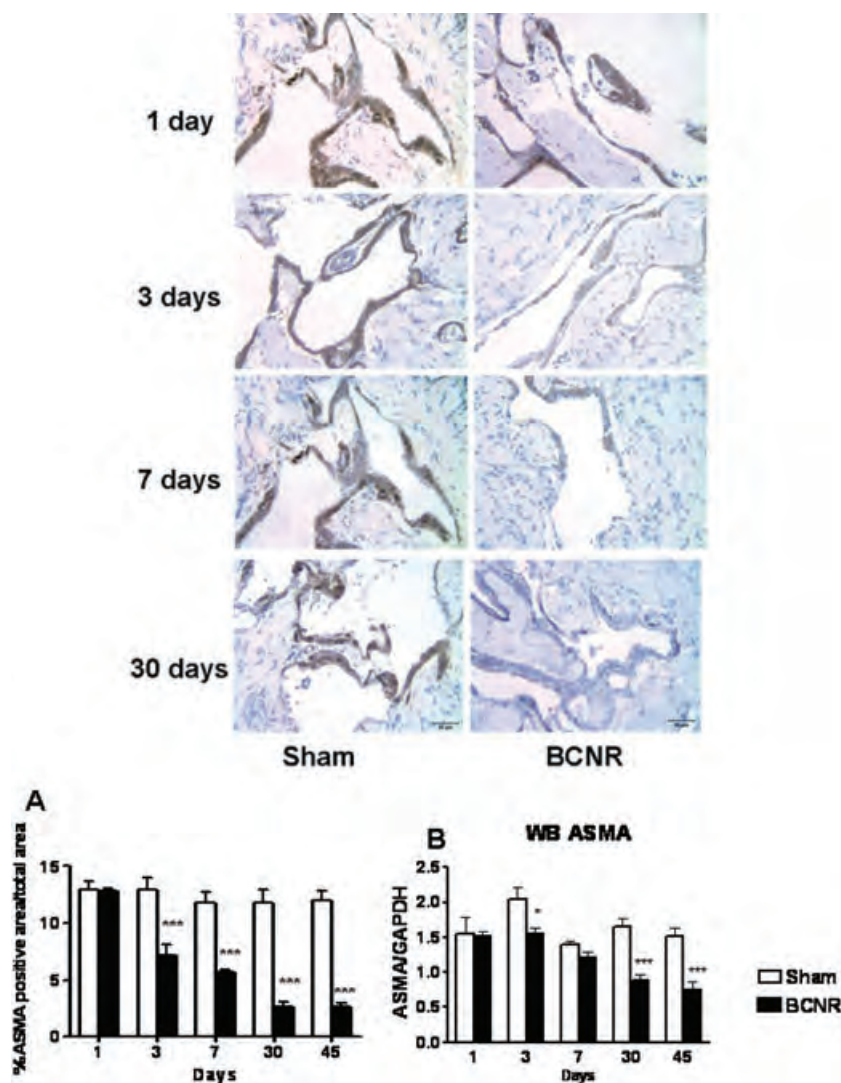
Figure 3 Time course of the effect of bilateral cavernosal nerve resection on the smooth muscle cell content in the rat corpora cavernosa. Penile corpora cavernosa sections adjacent to those presented on Figure 2 were immunostained for α-smooth muscle actin (ASMA) as a smooth muscle cells marker. Top: 400×, Bar = 50 μm. A, Quantitative Image Analysis of Asma expression by immunohistochemistry. B, ASMA Expression by western blot analysis on penile homogenate extracts. $*P < 0.05$; $***P < 0.001$. Sham = sham-operated animals; BCNR = animals subjected to nerve resection killed at 1, 3, 7, 30, and 45 days after surgery.

so evident, no quantitative determination was deemed necessary to corroborate the visual inspection. In contrast, no changes were appreciable in the immunohistochemical detection of eNOS, which was constrained to the endothelium lining of the corpora cavernosa lacunar spaces or cisternae (Figure 4B). This was confirmed by QIA.

In an even more marked contrast to nNOS decrease, iNOS immunostaining in the corpora of the BCNR rats started to increase by 10-fold at day 3, and continued to remain high throughout the study period, while it stayed almost undetectable in the sham-operated animals at all time periods (Figure 5, top). The quantitative determination indicated that iNOS expression reached a peak at 30 days, when there was about a 50-fold increase over both the sham-operated and the preinjury values (bottom).

The Reduction in SMC Occurring After BCNR Is Due to an Early Peak of Apoptosis That Initially Is Compensated by Increased Cell Proliferation But Later on Predominates Over This Process

TUNEL immunodetection assay revealed that by 1 day, and more so at 3 days following BCNR, there was a marked increase in apoptosis of cells in the corpora (not shown), and this was confirmed by QIA, which showed that the peak of apoptosis occurred at 3 days with a fivefold increase in the apoptotic index in the BCNR animals. This was followed by a gradual reduction, but still showing an over twofold higher apoptotic index at 45 days after BCNR (Figure 6, top).

When cell proliferation was measured by immunohistochemistry for PCNA, there was an intensification of cell proliferation at 1 and 3 dayspost-BCNR, but this level was subsequently
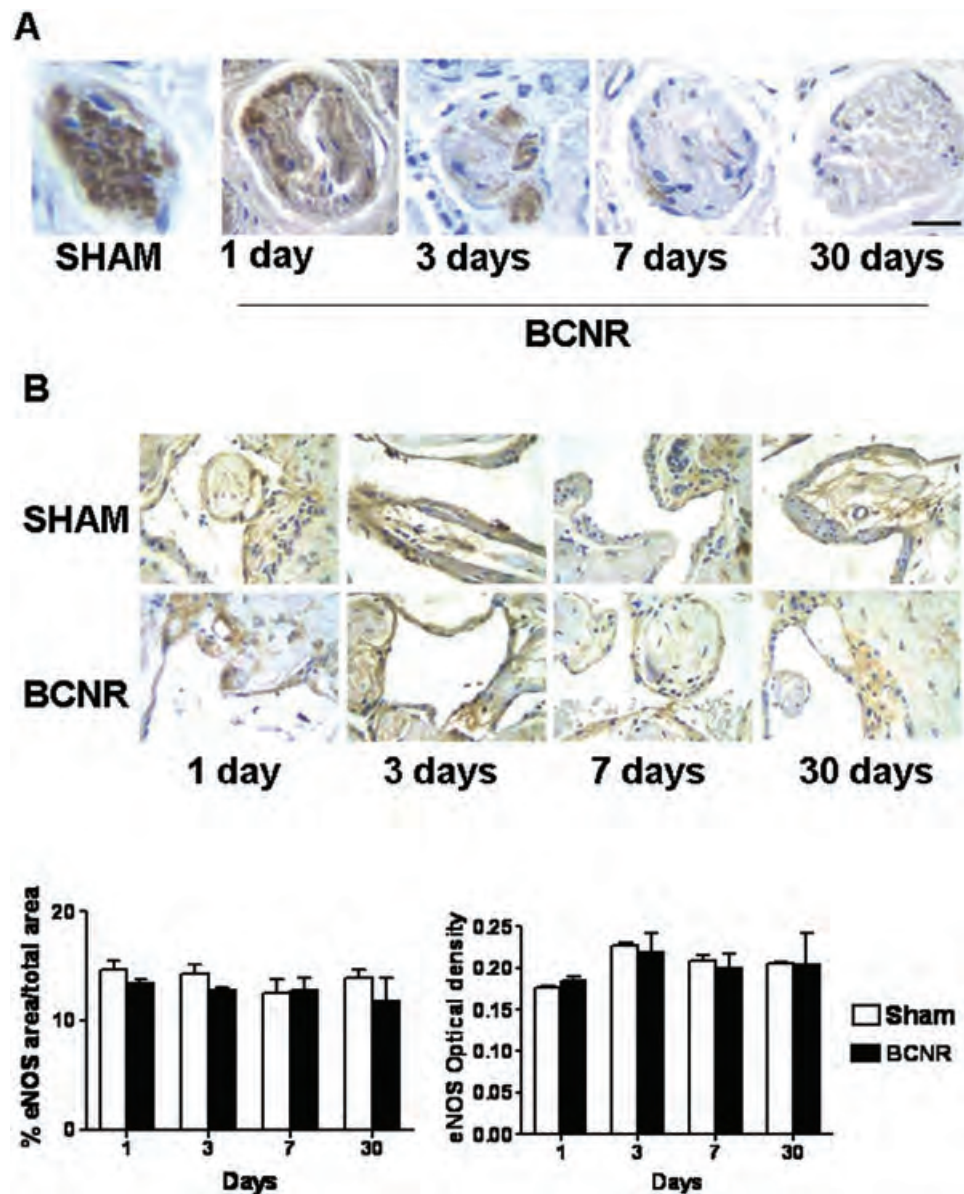
Figure 4 Time course of neuronal NOS (nNOS) expression after nerve resection in cavernosal nerve terminals and of endothelial NOS (eNOS) in the corporal enothelium. (A) penile sections adjacent to those presented on Figure 2 were immunostained for nNOS. Magnification: 400×, bar = 50 μm. (B) sections adjacent to those presented on Figure 2 were immunostained with an eNOS antibody. The expression of eNOS is not altered by nerve resection. Top: 400×, bar = 50 μm. Bottom: quantitative image analysis. Sham = sham-operated animals; BCNR = animals subjected to nerve resection killed at 1, 3, 7, 30, and 45 days after surgery.

reduced by day 7 postinjury to basal levels (Figure 6, middle). QIA showed that, as in the case of apoptosis, the cell proliferation peak occurred at 3 days, with a similar fivefold increase in PCNA staining, which decreased thereafter. Interestingly, at 30 and 45 days post-BCNR, the PCNA values in the BCNR groups were lower than in the control sham-operated animals. Because of the initial stimulation of cell replication, the ratio between the proliferation and apoptotic indexes in the corpora (bottom) remains around a value of 1 until 7 days after BCNR, with no significant differences between BCNR and the sham-operated rats. However, at both 30 and 45 days, there is a considerable reduction in PCNA due to the predominance of cell death over cell proliferation. This agrees with the time course for SMC content in Figure 3.
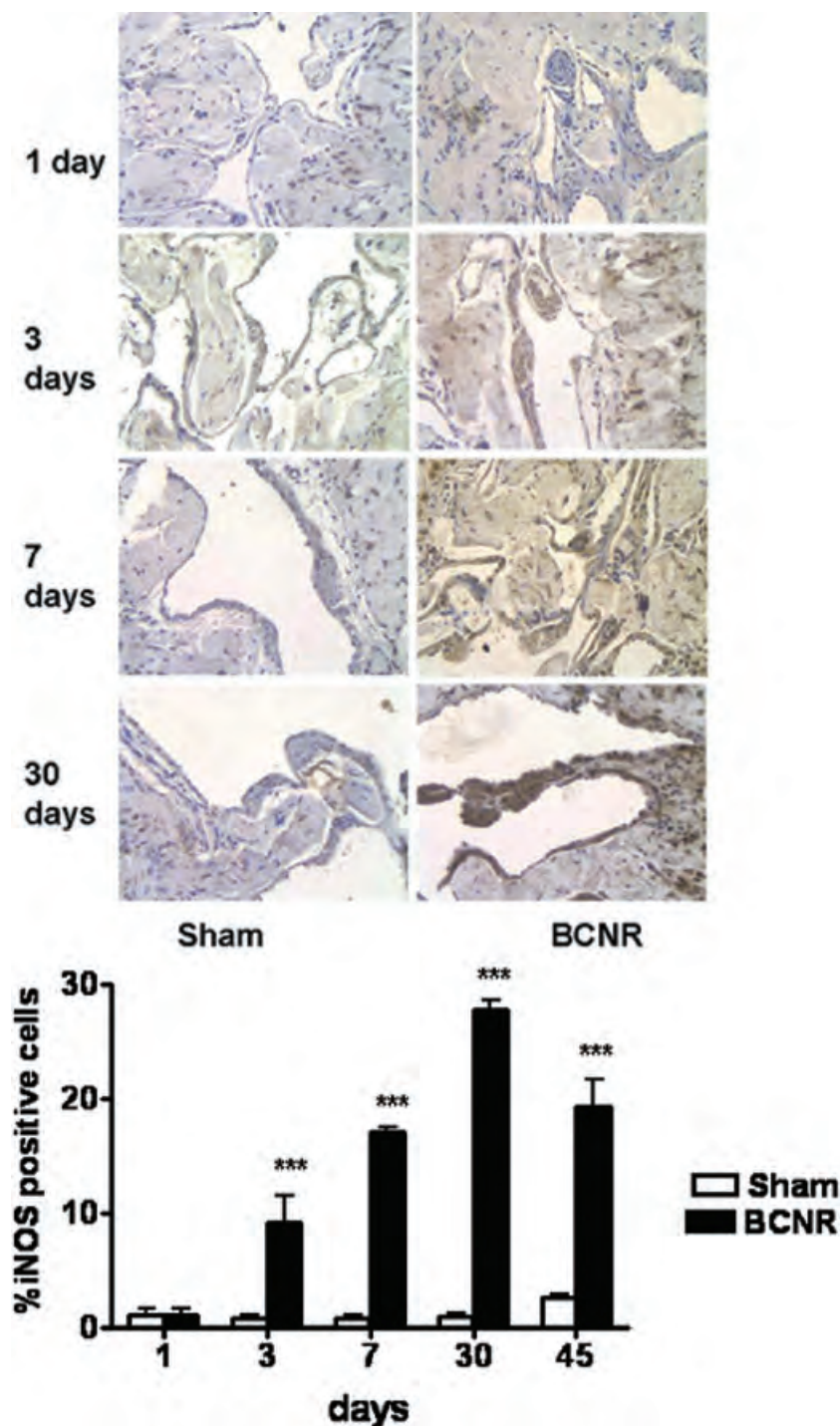
Figure 5 Time course of the effect of bilateral cavernosal nerve resection on the expression of inducible nitric nitric oxide synthase (iNOS) in the penile corpora cavernosa. Penile corpora cavernosa sections adjacent to those presented in Figure 2 were subjected to immunodetection for iNOS. Top: representative pictures (200×). Bottom: quantitative image analysis. ***$P < 0.001$. Sham = sham-operated animals; BCNR = animals subjected to nerve resection killed at 1, 3, 7, 30, and 45 days after surgery.

The western blot analysis of PCNA expression in total penile shaft homogenates (Figure 7) confirmed the decrease in PCNA staining seen by immunohistochemistry in the tissue sections of the corpora cavernosa of BCNR rats (Figure 6, middle panel). However, the levels of PCNA in the homogenates of the penile shaft (Figure 7) were inconsistently high at the two earliest time periods, probably reflecting the presence of tunical and corpus spongiosum tissue (not considered in the analysis of the tissue sections of Figure 7).
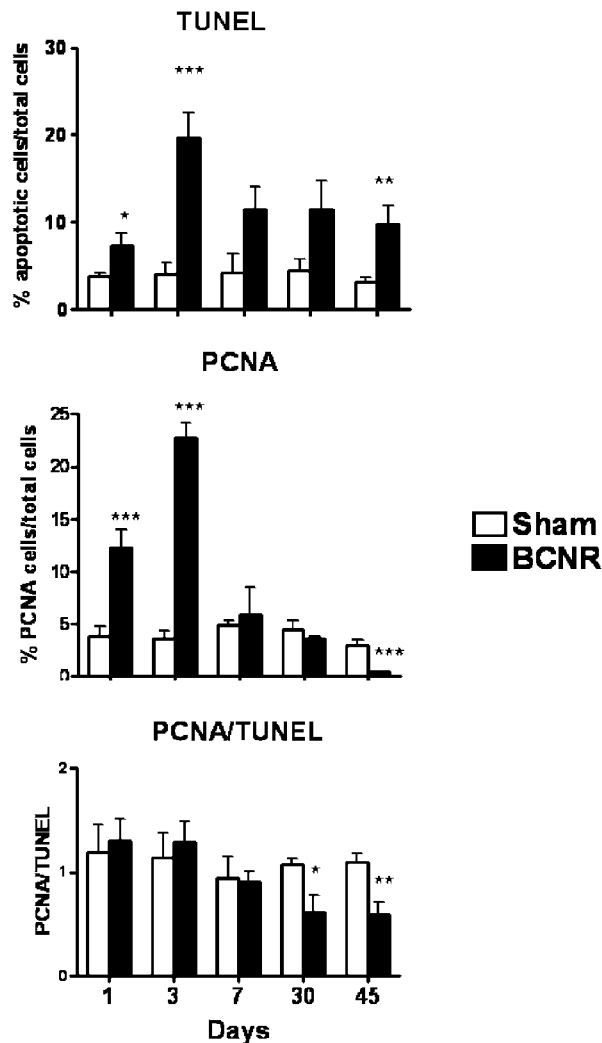
TUNEL

PCNA

PCNA/TUNEL

Figure 6 Time course of the effect of bilateral cavernosal nerve resection on the cell turnover in the rat corpora cavernosa. Penile corpora cavernosal sections adjacent to those presented on the preceding figures were subjected to TUNEL and proliferating cell nuclear antigen (PCNA) staining. Top: quantitative image analysis (QIA) for TUNEL. Middle: QIA for PCNA. Bottom: the ratio between the total area occupied by cells undergoing cell replication (PCNA+) and the apoptotic index obtained above was established for each animal, and then used to calculate means ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Sham = sham-operated animals; BCNR = animals subjected to nerve resection killed at 1, 3, 7, 30, and 45 days after surgery; Tunel = Terminal Transferase dUTP Nick End Labeling; SEM = standard error of the mean.

Discussion

The current results clarify the sequential events that lead to the development of CVOD in the rat following cavernosal nerve damage. The assumption is that CVOD or venous leakage occurs because the SMC mass in the corpora is impacted

in such a way that it cannot achieve sufficient relaxation to attain an intracorporeal pressure high enough that can compress the subtunical veins as they egress from the tunica albuginea of the penis. Normally, this is evident by a decrease in the SMC content, together with an increase in tissue fibrosis within the corpora.

The absolute amount of corpora smooth muscle that only drops significantly at day 30, but not at 7, approaching the value at day 45, appears to be more critical for corporal compliance and venous occlusion than the smooth collagen content ratio that falls down earlier. This interpretation would explain the fact that the significant increase in drop rate occurs at day 30 but not at 7 days, thus implying that a certain threshold in the corporal smooth muscle content combined with collagen deposition may be needed, below which the functional impairment would become evident.

The absence of a parallel significant decrease in the papaverine response at day 30 (despite the trend seen on Figure 1) may be due to the relatively high papaverine dosage (100 µL of 20 mg/mL solution, which is approximately 5 mg/kg. body weight) used in this study. This may be excessive to detect a marginal CVOD, based on the erectile response to the drug. However, we have recently conducted a papaverine dose/response titration curve during DIC in the rat, and we have found that 15 mg/mL of papaverine is an optimal concentration (or 3.8 mg/kg) for performing DIC, and this intracorporal dose will be used in the future.

Therefore, if the hypothesis of the decrease in the SMC content, together with an increase in tissue fibrosis within the corpora is correct, then apoptosis should occur first, followed by an observed decrease in the corporal smooth muscle content in combination with an increase in tissue fibrosis before CVOD becomes evident. Indeed, in our animal model of BCNR, the process of apoptosis is apparent 24 hours following the neural injury, an observation that has been previously reported by others [21–24]. What our data does show for the first time is that this apoptotic process peaks around 3 days following BCNR, and, although there is a slight decrease from this peak level seen after day 3, the level of apoptosis continues to remain elevated up to the end of the experiment, which was 45 days after BCNR.

The data confirm the observation of previous investigators [20–23] that programmed cell death is apparent as early as 1 day after the onset of
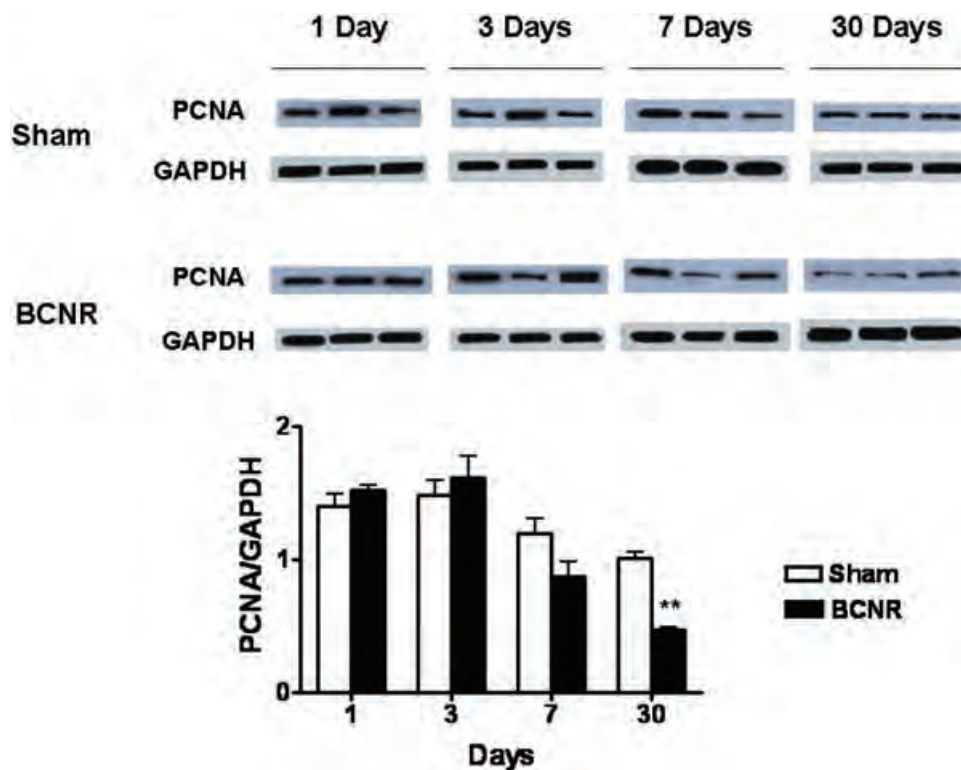
Figure 7 Corroboration of the proliferating cell nuclear antigen (PCNA) immunostaining by western blot. Homogenates from corpora cavernosa tissue were subjected to western blot analysis with the same antibody used for Figure 6. **$P < 0.01$. GAPDH = glyceraldehide-3-phosphate dehydrogenase. Sham = sham-operated animals; BCNR = animals subjected to nerve resection killed at 1, 3, 7, 30, and 45 days after surgery.

the neural injury. In addition, while the peak for these proapoptotic processes occurs by day 3 following the neural injury, there also appears to be a considerable increase in cell proliferation within the trabecular tissue around the cisternae, a finding that has not been previously reported, and one that may represent an attempt by the tissue itself to counteract apoptosis. Thereafter, cell proliferation, by drastically declining already at 7 days, becomes insufficient to counteract the much slower decline in apoptosis. As a result, the imbalance between both processes manifests at 30 days, agreeing with the earliest period, where there is a net loss of SMC. Since the ratio of the SMC to collagen decreases significantly, and rather drastically at 7 days after BCNR, but the content of the SMC decreases much earlier, at 3 days, which coincides with the peak in apoptosis, it may be concluded that collagen deposition is intensified after the SMC loss, and that therefore the reduction of the cellular compartment precedes the onset of fibrosis. It is the net loss of SMC that appears to trigger the first manifestation of CVOD that occurs 30 days after BCNR.

The reduction of nitrergic nerve terminals that are clearly distinguishable from the dorsal nerve and may be ascribed topologically to the cavernosal nerve, is evident as early as 1 day after BCNR. This suggests that Wallerian nerve degeneration exacerbated throughout the 45-day period is most likely responsible for the changes observed in the corpora cavernosa SMC. Most interestingly and somewhat surprisingly was the lack of changes in the content of eNOS, thereby suggesting that the endothelium is not considerably affected by BCNR. This indicates that: (i) eNOS-dependent endothelial dysfunction may not be elicited by neuropraxia and is not involved in CVOD, which appears to result mainly from corporal SMC loss and fibrosis; and (ii) in the absence of nNOS, eNOS cannot per se produce sufficient nitric oxide as to sustain the papaverine-induced production of cGMP caused by the unspecific PDE inhibition exerted by the drug [25]. However, since neither endothelial function nor eNOS activity has been determined, it is not possible to rule out a possible functional impairment of the endothelium after BCNR despite unaltered expression of eNOS.

Perhaps the most intriguing observation is the time course of iNOS induction by BCNR, which seems to follow the nNOS decrease in the nitrergic nerves but peaks at 30 days. This is long after apoptosis has reached a maximum at 3 days, thus ruling out the possibility that this cell death is triggered by nitric oxide from iNOS, a compound that is usually considered as proapoptotic [26,27]. However, there is evidence that nitric oxide can in fact be antiapoptotic according to tissue and physiological conditions [28]. Alternatively, this sustained increase of iNOS expression may be responsible for the observed reduction of the compensatory cell proliferation in the corpora after BCNR, based on the fact that both nitric oxide and cGMP are considered to be antiproliferative for the SMC in the arterial media [29]. However, this possibility appears to be ruled out by our previous results with N6-(1-iminoethyl)-L-lysine dihydrochloride (L-NIL), an inhibitor of iNOS activity [11,15]. At least at 45 days after BCNR, a steady iNOS inhibition by daily oral L-NIL significantly reduced the SMC/collagen ratio, thus suggesting that iNOS is acting by protecting the SMC or inhibiting collagen deposition, which would be in agreement with the cardioprotective effects of nitric oxide, cGMP, and iNOS on cardiomyocytes during ischemia reperfusion pre or postconditioning [30–32]. iNOS may not only be produced by smooth muscle cells, since macrophages and interstitial fibroblasts are also known to express this protein upon induction. No colocalization studies for iNOS and ASMA were performed in this work, and therefore it cannot be ruled out that iNOS synthesis in the corpora occurs also in cell types other than the smooth muscle cells. In addition, it is not surprising that a steady increase in iNOS would occur in the presence of a sustained decline in the overall content of the putative cell type where iNOS is induced, since iNOS expression is due to transcriptional stimulation, which, by a steady increase within each cell and the cumulative production of nitric oxide, can substantially exceed the rate of cell loss.

However, since CVOD and fibrosis do develop in BCNR despite the steady iNOS production, this process is apparently insufficient to counteract the factors that trigger "corporal dystrophy" (fibrosis and SMC loss), a term that we propose as analogous to skeletal muscle dystrophy.

This leads to the fundamental question regarding which factors triggered by the neuropraxia are responsible for causing corporal SMC dystrophy. The most likely is the interruption of the secretion of neurotrophins, which, in addition to their effects on neural tissue [33,34], are postulated to stimulate smooth muscle hyperplasia, particularly in the respiratory airways and the intestine [35,36]. This depletion may cause the downregulation of SMC proliferation triggered by a spontaneous defense mechanism against neuropraxia. Conversely, the induction of cytokine release, mainly TNFα and TGFβ1, that are proapoptotic and fibrotic factors and activate the proteasome ubiquitin proteolytic pathway, is a recognized feature of Wallerian degeneration [37], and it underlies at least in part the skeletal muscle atrophy subsequent to denervation [38,39]. However, the lack of neuromotor discharge and activity may also be an essential factor in this atrophy.

Irrespective of the mechanism that triggers fibrosis and SMC loss subsequent to cavernosal nerve damage, three things became obvious through this work. First, that it is the early histopathological impairment within the corpora smooth muscle that leads later to the functional impairment, CVOD. It may require a certain threshold in the smooth muscle/collagen ratio that below that threshold, the functional impairment becomes evident Therefore, in the clinical setting, an early therapeutical intervention to reduce apoptosis of the corporal SMC or sustain their initial proliferation response, would be warranted, e.g., immediately after radical prostatectomy. Second, since iNOS induction appears to be an endogenous antifibrotic and protective response on the smooth muscle, the early therapy may be based on pharmacological agents that mimic this process, such as the continuous long-term administration of PDE5 inhibitors we have studied in rats [10–13,33,40], or of nitric oxide generators [41,42], or in men for a combination of both types of compounds [43]. Such a therapeutic modality may be accompanied with neurotrophin administration, such as BDNF [34], in an attempt to restore the anabolic signals to the smooth muscle that endogenous neurotrophic factors are no longer mediating. Third, since our experimental model of BCNR, where the cavernosal nerves are both completely resected, may not be very representative of the cavernosal nerve injury that may occur with pelvic surgery, in which the nerves may only be partially damaged, it is possible that axonal regeneration, which does not seem to occur after BCNR within the time course of this study, may occur with radical pelvic surgery, particularly with the nerve sparing

procedures. As such, the treatments described above to prevent the histological changes in the corpora may also be efficacious in stimulating axonal regeneration.

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BASES CELULARES Y MOLECULARES DE NUEVOS ENFOQUES TERAPEUTICOS PARA LA ENFERMEDAD DE LA PEYRONIE Y LA DISFUNCION ERECTIL

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Indice de contenidos:

1.- Introducción

A pesar de los más de 250 años transcurridos desde su descripción clínica inicial, la enfermedad de La Peyronie, o de "Peyronie' **(Peyronie's disease o PD")** como se la identifica más comúnmente en la literatura anglosajona y en PubMed, permanece como una "enfermedad huérfana" debido a la carencia de una terapia no quirúrgica efectiva **(1).** Ello es sorprendente cuando se considera que la formación de la placa fibrótica en la túnica albugínea del pene, que caracteriza a esta condición y lleva a la deformación del pene erecto, frecuentemente da dolor y disfunción eréctil **("erectile dysfunction o ED"),** afecta seriamente la calidad de vida y tiene una alta prevalencia (estimada entre el 5 y el 9% de la población masculina) **(1).** Los agentes terapéuticos usados clínicamente tienen una eficacia muy baja o debatible, restringida a modestos efectos sobre la curvatura del pene y el dolor asociado en la etapa inicial **(2)**. El fracaso en lograr un tratamiento eficaz resulta en parte del escaso interés en financiar la investigación pertinente por parte de las agencias gubernamentales y empresas farmacéuticas. Aunque esto aparentemente contrasta con el apoyo económico que llevó a la aplicación exitosa de los inhibidores de la fosfodiesterasa 5 **("phosphodiesterase 5 inhibitors, PDE5i")** para la ED. En el fondo el abordaje actual

de ambos procesos coincide, ya que esta terapia en su modalidad actual es meramente paliativa (induce un efecto a corto plazo: la erección) y no afecta la histo-fisiopatología subyacente, o sea que consiste en una terapia no curativa **(3-5).**

A pesar de esta realidad, muchos laboratorios de investigación básica traduccional (orientada a la clínica) continúan investigando y proponiendo enfoques terapéuticos novedosos para ambos desordenes, emergentes de conceptos desarrollados en la última década sobre un común denominador histo-fisiopatológico: **la fibrosis tisular progresiva**. En la PD este proceso afecta la túnica albugínea, es relativamente rápido y lleva a una o varias placas fibróticas localizadas; En cambio, en el tipo más común de ED, la disfunción córporo-veno-oclusiva **("corporal veno-occlusive dysfunction o CVOD")**, es generalmente lento, difuso, y afecta la musculatura lisa de los cuerpos cavernosos **(6)**. Las excepciones más significativas son la CVOD subsiguiente a prostatectomía radical, donde la neuropraxia resultante del daño a los nervios cavernosos produce un impacto abrupto y acelerado en la musculatura lisa corporal **(3-5;** ver más adelante), o la ED arteriogénica donde un lento proceso de fibrosis afecta predominantemente la musculatura lisa de la túnica media de la pared arterial de los vasos que irrigan el pene **(7).** Otras fibrosis de los cuerpos cavernosos, como la subsiguiente a priapismo o la inducida ocasionalmente por drogas inyectadas en el pene, comparten algunas de estas semejanzas o diferencias, pero no son tratadas en este capítulo.

Por conveniencia, las abreviaturas a lo largo del texto respetan, salvo las excepciones indicadas, el estilo internacional, es decir las correspondientes en el idioma inglés.

2.- Bases celulares y moleculares de los procesos fibróticos en el pene.

Las bases moleculares y celulares de la placa fibrótica en la PD se asemejan a las presentes en la CVOD y la ED arteriogénica, aun cuando hay importantes diferencias. En la PD, el factor de transformación y crecimiento celular beta 1 **("transforming growth factor beta 1 o TGFβ1),** junto con otras citoquinas y, particularmente, el inhibidor 1 del activador de plasminógeno **("plasminogen activator inhibitor 1, o PAI-1")** son fundamentales en la iniciación y progresión de la placa **(8,9).** Uno de sus efectos es inducir la generación de **miofibroblastos**. Estas son células con un fenotipo hibrido entre fibroblastos y células musculares lisas, que son clave para el depósito de colágeno y de matriz extracelular, tanto en circunstancias normales de cicatrización de heridas, como en condiciones anormales como en la placa de PD y otras fibrosis **(Fig. 1).** La diferencia es que en el proceso normal los miofibroblastos desaparecen por apoptosis (muerte celular programada) después de cumplir su función, mientras que en la fibrosis persisten multiplicándose y en actividad secretoria.

La combinación de la liberación de TGFβ1 activo y la generación de miofibroblastos es común a muchos procesos fibróticos difusos que afectan el riñón, hígado, pulmón, corazón, y otros órganos en

diversas patologías **(10),** pero su papel en la fibrosis de los cuerpos cavernosos en la CVOD no es claro**.** En cambio, la generación de las especies de oxígeno reactivo **("reactive oxygen species o ROS"),** durante el "stress" oxidativo es un factor común en la PD, CVOD y ED arteriogénica, así como en otras fibrosis. Lleva a la síntesis exagerada de colágeno y matriz extracelular y, en el caso de la CVOD y ED, a la pérdida de células musculares lisas por apoptosis **(6,8,9).** Procesos tales como la creación de uniones cruzadas en el colágeno por los "productos de glicosilación avanzada terminal" ("advanced glycation end-products", o **"AGE")** inducidos por la hiperglucemia en la diabetes no controlada **(11),** y la inactivación o disminución de la síntesis de las metaloproteinasas ("**MMPs")** (enzimas que degradan el colágeno) permiten la acumulación de esta proteína **(12).**

Tanto en la PD humana como en sus modelos experimentales se ha establecido que un factor etiológico es el **microtraumatismo** en el pene erecto durante la actividad sexual, que permite la extravasación de albúmina, fibrinógeno y otras proteínas, que se depositan en la túnica albugínea e inician un proceso inflamatorio agudo **(1,8,9,13)**. La persistencia de fibrina, generada a partir del fibrinógeno, es debida a la liberación de PAI-1, que inhibe la fibrinolisis y, junto con citoquinas como IL-6, lleva a una inflamación crónica, y además induce la expresión de TGFβ1 **(14).** Este factor, combinado con la acumulación de ROS, estimula la aparición y persistencia anormal de miofibroblastos y la síntesis de colágeno. Causa la iniciación de la placa, que progresa a expensas de un crecimiento y endurecimiento paulatinos. Por un lado, por la creación de uniones cruzadas en el colágeno y, por otro, por la calcificación u osificación, observable en un 15-20% de los casos. Este último proceso es debido a la generación de osteoblastos a partir de los miofibroblastos, o de células madre presentes en la túnica albugínea **(15)**.

Estos mecanismos han sido elucidados en esta última década (**1,8,9,16**) a través de estudios realizados en**:**

a) la placa fibrótica humana en comparación con la túnica albugínea normal;

b) modelos animales basados en la generación en la túnica albugínea de la rata o el ratón de una lesión que se asemeja al PD humano, mediante la inyección de ya sea TGFβ1, péptidos relacionados, o virus que lo expresan (simulando la liberación inicial de citoquinas), o bien fibrina que induce TGFβ1 y PAI-1 (simulando el proceso de microtrauma);

c) cultivos de células de la placa de PD y de túnica albugínea humanas y sus contrapartes de los modelos animales, con la identificación de células madre.

Uno de los aspectos de más interés que emergió de estos estudios es que tanto en la PD como en la CVOD el proceso inflamatorio/fibrótico conlleva la expresión de la sintetasa inducible del óxido nítrico **("inducible nitric oxide synthase, iNOS, o NOS II"),** que no ocurre en general en los tejidos normales **(6,17,18).** En condiciones patológicas iNOS actúa como una respuesta inflamatoria, inmunológica, o

antiinfecciosa, que lleva a la apoptosis de células extrañas como bacterias o células cancerosas. Sin embargo, en la PD, la CVOD, la arteriosclerosis asociada con el envejecimiento y la diabetes, la inducción endógena sostenida de iNOS principalmente por fibroblastos, miofibroblastos, y macrófagos, produce niveles moderadamente altos y también sostenidos de óxido nítrico que, al estimular la guananil ciclasa, lleva a una producción continua de GMP cíclico **(cGMP).**

Tanto el óxido nítrico como el cGMP actúan como un mecanismo de defensa antifibrótico; y, en el caso del óxido nítrico, también reducen el nivel de ROS a través de su eliminación en una reacción que produce peroxinitrito, o sea reduce el "stress" oxidativo. Ello se ha podido demostrar en los modelos de PD y ED **(6,13,17,20)** sobre la base de que reduciendo la actividad de iNOS con administraciones diarias y crónicas de un inhibidor especifico (L-NIL), o bloqueando el gen en los ratones transgénicos "knock-out", se aumenta considerablemente la fibrosis, el numero de fibroblastos, y el stress oxidativo. Alternativamente, la inducción de iNOS por transferencia génica inhibe estos procesos. Una situación similar ha sido demostrado en varios procesos fibróticos en otros órganos **(ver 17).**

Finalmente, estudios recientes de comparación de la expresión de múltiples genes con los procedimientos denominados "DNA microarrays", particularmente en PD, han permitido postular que la placa de PD está en un permanente recambio celular y molecular, y no es un proceso estático irreversible **(21,22).** Ello se infiere por el aumento de la expresión en la placa de genes vinculados no sólo con la inducción de iNOS, sino con otros procesos de defensa antifibrótica, como la inducción de ciertas metaloproteinasas **(MMPs)** o colagenasas (que realizan la digestión del colágeno), o pro-apoptóticos (que reducen la población de miofibroblastos). En consecuencia, es potencialmente factible modificar el balance de síntesis/degradación de las fibras colágenas o de generación/apoptosis de miofibroblastos con intervenciones farmacológicas; que, de tener éxito, podrían reducir el tamaño y "ablandar" la placa.

3.- Fisiopatología de la fibrosis y nuevas estrategias farmacológicas para el tratamiento de la Enfermedad de La Peyronie.

Basado en estos conceptos, la premisa básica de los nuevos conceptos terapéuticos (que superen la mera aplicación empírica de fármacos de dudosa acción antifibrótica) es tratar de cambiar el balance del recambio celular y molecular de la placa de PD para lograr:

a) el predominio de la degradación del colágeno sobre su síntesis **(13);**

b) el predominio de la apoptosis de los miofibroblastos sobre su replicación **(13);** o

c) la inhibición de la producción de miofibroblastos y osteoblastos a partir de células madre en la placa **(15).**

Varios enfoques están siendo estudiados para reducir la deposición de colágeno y el crecimiento de la placa en a) y b):

- En primer término, están los **PDE5i** y algunos inhibidores generales de PDE **(9)** que, debido a su uso clínico y su potencial como tratamiento "curativo" de la CVOD, serán discutidos al final de esta sección.

- Están emergiendo varias estrategias de **terapia génica**. Los inhibidores, anticuerpos o shRNAs (que inhiben la traducción de proteínas especificas) bloquean genes, o sus productos críticos para el proceso fibrótico. Mediante "DNA microarrays" estos genes fueron identificados al comparar cultivos celulares de la placa de PD humana con los de la túnica albugínea normal, en condiciones basales o pro-fibróticas (con TGFβ1) **(22).** Esta "firma transcripcional" reveló un fenotipo de las células de PD que es altamente replicativo, pro-fibrótico, pro-inflamatorio, y miogénico (miofibroblastos)**.** Ello sugiere una nueva terapia para PD basada en inhibir por vía intra-lesional genes como:

    a) IGF1, EDNRB, EGR2 (proliferación de miofibroblastos);

    b) MYF5, ACTC1 (generación de miofibroblastos);

    c) PAI2, PAI10 (fibrosis); o

    d) TNFα, IL11, IL6 (inflamación).

- Adicionalmente, aparecen como plausibles y de aplicación más inmediata:

    a) El **bloqueo de la señalización por TGFβ1**:

    - mediante un inhibidor de su receptor, que ya demostró en el modelo de PD en la rata ser efectivo en reducir la curvatura, el tamaño de placa y el depósito de colágeno **(23),**

    - o bien por un agente que neutraliza al TGFβ1, como la **decorina**, que también tuvo eficacia en la fibrosis de los cuerpos cavernosos inducida por diabetes **(24),**

    b) Los **nuevos medicamentos antiinflamatorios contra TNFα,** como el **infliximab**, utilizado en la enfermedad de Crohn **(25).**

- Otro enfoque para reducir el depósito de colágeno se basa en extrapolar a la PD resultados experimentales prometedores para el tratamiento oral de largo plazo de la fibrosis de los cuerpos cavernosos y CVOD, usando fármacos aplicados en la clínica en otros desordenes no relacionados con la fibrosis, tales como:

    a) **antioxidantes** (con el objeto de reducir el "stress" oxidativo) que resulten más efectivos en PD que la vitamina E, tales como el inhibidor de xantino óxidoreductasa, alopurinol (gota, dermatología) **(17)**;

    b) **tiazolidenedionas,** tales como la **pioglitazona** (Actos) usado para el tratamiento de diabetes tipo II, que aparte de ser efectivos contra CVOD, son también antiinflamatorios y antifibróticos sobre el riñón en la nefropatía diabética **(26-29).**

- Finalmente, una cantidad de agentes que son prometedores en el tratamiento clínico de la fibrosis renal, hepática, y p ulmonar, están siendo considerados para pruebas intralesionales u orales en los modelos animales de PD. Ellos incluyen agentes que inhiben la síntesis de colágeno o neutralizan indirectamente el TGFβ1 actuando localmente en el tejido, tales como:

a) **pirfenidona (30,31);**

b) bloqueadores de los receptores de angiotensina II y antagonistas del receptor de endotelina, como **bosentan (32)**.

- Paradójicamente, el blanco más directo para reducir la placa de PD, o s ea la inducción de la degradación de las fibras de colágeno, no ha sido sometida a estudios sistemáticos preclínicos en modelos animales. Los trabajos preliminares, pero no suficientemente validados, de Gelbard con colagenasa hace más de dos décadas han llevado a nuevas pruebas clínicas, que están en curso, utilizando **colagenasa de** *Bacillus Clostridium* **(33,34)**. Sin embargo, hay problemas potenciales que deben ser resueltos con estudios preclínicos, tales como:

a) la posibilidad de que esta colagenasa no sea la más adecuada para reproducir la mezcla de MMPs presentes en la túnica albugínea normal y/o para eficazmente degradar la mezcla de colágenos I, III, y XII que caracterizan el cultivo celular de la placa de PD **(21,22),** y posiblemente la placa en si misma**;**

b) el riesgo de que el colágeno de la túnica albugínea normal sea más sensible a este tratamiento que el "endurecido" por uniones cruzadas en la placa, y la colagenasa bacteriana termine debilitando la tunica normal y causando CVOD.

Por esta razón, son necesarios nuevos estudios de laboratorio enfocados en la comparación, por un lado, de la expresión de todos los tipos de colágenos y MMPs, así como los inhibidores de MMPs llamados **inhibidores titulares de MMPs** ("**tissue inhibitors of MMPs, o TIMPs");** y, por otro lado, de las actividades enzimáticas endógenas de MMPs. De esta forma sería posible definir la MMP más adecuada para digerir selectivamente la placa de PD.

- Retomando el enfoque de inhibir el depósito de colágeno, estudios en el modelo de PD en la rata, demostraron que un **inhibidor inespecífico de PDE** pero que afecta predominantemente a l a PDE4, la **pentoxifilina**, dado en administración oral continua y prolongada, es efectivo en prevenir el desarrollo de la placa **(35).** Ello fue confirmado por la inhibición en cultivos celulares de los efectos pro-fibróticos del TGFβ1 **(36)**. Los estudios clínicos en PD son sin embargo contradictorios y uno de ellos concluyó que la pentoxifilina es moderadamente efectiva en reducir la curvatura del pene y el volumen de la placa en pacientes en una etapa inicial de PD **(37,38),** pero en general se basan en un numero pequeño de casos y no están adecuadamente validados.

El estudio pionero en ratas ya citado **(35)** fue también el primero en concluir que la administración diaria oral a largo plazo de **sildenafil** (Viagra) ejerce efectos aun más pronunciados, y lo mismo ocurría con **L-argina**, el substrato de NOS para producir óxido nítrico. Se postuló que la elevación sostenida de los niveles de óxido nítrico por L-aginina, y el subsiguiente incremento de su agente efector, el cGMP, o la preservación de los niveles de cGMP por sildenafil actuaban como antifibrótico, reduciendo el contenido de colágeno y el numero de miofibroblastos. Ello fue confirmado dando **vardenafil** (Levitra) oral por largo plazo en el agua de beber, tanto para prevenir como para revertir el desarrollo de la placa, o incluso en forma discontinua por administración retrolingual **(39).** El mecanismo propuesto se presenta en la **Fig. 2.**

En conclusión, los estudios de tratamientos con más inmediato potencial clínico, basados en las citadas pruebas en modelos animales, apuntan a la administración sostenida y a largo plazo de PDE5i, particularmente en la etapa inicial de PD; pero se requieren ensayos clínicos controlados. Sin embargo, estos agentes per se podrían no ser suficientemente efectivos en el hombre, y quizás convendría investigar regimenes de combinación con otros fármacos que, como la pirfenidona o la pioglitazona, están ya en el armamentario clínico. De todos los potenciales enfoques experimentales que aun no han sido investigados en pruebas preclínicas específicamente para PD, quizás el más prometedor es la selección de una MMP en función de la expresión de colágenos y de la relación MMP/TIMP para potenciar la degradación selectiva de la placa.

4.- Estudios experimentales sobre la fisiopatologia y terapia de la fibrosis en los cuerpos cavernosos y la disfunción córporo-veno-oclusiva.

Los PDE5i se utilizan en la práctica clínica, desde su comercialización a mediados de la década pasada, para facilitar la erección en pacientes con ED, en regimenes de administracion oral "**a demanda**" ("on demand") **(40,41).** Ello conlleva un tratamiento esporádico y paliativo no orientado hacia el tratamiento ni la prevención de la patología subyacente, en dosis pre-establecidas para inducir un efecto vasodilatador en los cuerpos cavernosos del pene que produzca una erección rígida **(42).** El mecanismo de acción operativo en este paradigma estándar es el bloqueo transitorio de la degradación del cGMP en los cuerpos cavernosos del pene para mantener su concentración tisular en los altos niveles producidos durante la estimulación sexual, mediante la inhibición de PDE5 que lo degrada **(43).** Ello relaja la musculatura lisa corporal inhibiendo su tono mediante la reducción del calcio intracelular. Es decir, una vasodilatación que promueve la tumescencia, y eventualmente la erección, mientras el cGMP continúe elevado. Durante la actividad sexual, el cGMP es producido por la estimulación de la guanilil ciclasa soluble (**"sGC"**) de la musculatura lisa por el óxido nítrico producido por la NOS neuronal (**nNOS o NOS I),** al nivel periférico en los terminales nerviosos de los cuerpos cavernosos. Si no hay liberación de

óxido nítrico por falla de la libido o carencia del estimulo sexual no hay elevación del cGMP y en consecuencia no hay erección, lo que evita en teoría y práctica que los PDE5i produzcan una tumescencia o erección espontánea o un priapismo sostenido en ausencia de dicho estimulo**.**

Por 15 o más años esta administración oral de los PDE5i "a demanda" ha constituido el único modelo empleado, y el objetivo del tratamiento con estos agentes ha sido inducir la erección. El aliviar o curar la histo-fisiopatología que afecta los cuerpos cavernosos (fibrosis y pérdida de células musculares lisas) ni siquiera se consideraba, a pesar de que ésta es la causa de la impotencia vasculogénica (la más prevalente en el hombre) **(6).**

Recientemente, los urólogos y sexólogos han comenzado a considerar que algo debería hacerse para prevenir al menos este proceso en un tipo de ED donde el periodo del daño histológico en los cuerpos cavernosos que afecta la musculatura lisa es circunscrito y conocido, ya que ocurre inmediatamente tras la prostatectomía radical como se ha visto en el capítulo correspondiente de este tratado **(44-46).** El iniciador de este daño es la neuropraxia de los terminales de los nervios cavernosos que se afectan durante la cirugía debido a su sección, tracción, contusión, isquemia por daño vascular, injuria térmica, o inflamación por trauma quirúrgico **(3).** Esta condición se presta entonces para planear una intervención farmacológica con PDE5i conceptualmente diferente de la mera inducción de la erección, que esta orientada a prevenir o contrarrestar en una etapa inicial la ED per se, dentro de la nueva modalidad llamada "rehabilitacion del pene" ("penile rehabilitation") **(4)**.

El argumento más simple que se ha utilizado en estos y otros casos de ED para usar un nuevo régimen farmacológico para la administración crónica y continua de los PDE5i **(46-50)** se basa en que si la impotencia orgánica es por definición una condición crónica progresiva, el clínico debería tratarla crónicamente, o sea administrar estos fármacos no esporádicamente para inducir una erección, sino a largo plazo y en forma continua para prevenir o revertir el daño a los cuerpos cavernosos. En el caso de la prostatectomía radical **(3,4,46-50),** la neuropraxia derivada del potencial daño a los nervios cavernosos, particularmente cuando no se aplica la técnica con "**preservación de bandeletas nerviosas bilateral**" ("bilateral nerve sparing radical prostatectomy" o "BNSRP"), impacta gradualmente y en forma severa a la musculatura lisa corporal. Ello causa CVOD, o sea que no solamente existe el riesgo de una ED neurogénica por la neuropraxia per se, sino la resultante ED vasculogénica**.** Esta última puede ser de tal magnitud que ni los PDE5i administrados oralmente "a demanda", ni la administración local intracavernosa de prostaglandina E1, trimix ni otros vasodilatadores resulta eficaz para inducir la erección.

La rehabilitación del pene farmacológica que actualmente se considera en la clínica se basa en el tratamiento en forma crónica y continua, desde el momento más temprano posible tras la intervención quirúrgica. Persigue esa pauta de administración:

a) Tratar con PDE5i la disfunción vasculogénica o CVOD, pero no para inducir una erección a voluntad sino para combatir el proceso patológico que afecta la respuesta de relajación de los cuerpos cavernosos al NO/cGMP **(3,4,46,50);** y/o,

b) Tratar la disfunción neurogénica con agentes neuromoduladores (neurofilinas) que promuevan la regeneración de los axones neuronales dañados por la operación **(50,51).**

La opción "a" es la que tiene las mayores perspectivas de éxito teniendo en cuenta la disponibilidad clínica, lo relativamente bien tolerados que son estos fármacos y el hecho de la reciente autorización de la FDA para administración diaria y continua del tadalafil (Cialis) **(49).** Sin embargo, existen una cantidad de requisitos a cumplir para asegurarse que esta estrategia es segura y eficiente en términos clínicos; y, fundamentalmente, para entender el potencial mecanismo de acción para evitar fracasos. Este nuevo mecanismo es derivado de los estudios experimentales de laboratorio que actuaron de pioneros para proponer la nueva modalidad terapéutica de protección del músculo liso en los cuerpos cavernosos **(5,20,52,54)** y difiere radicalmente de la implícita en la extrapolación un tanto simplista de una "terapia crónica para una condición crónica". Esta visión simple, en general, acepta el mismo mecanismo de vasodilatación transitoria, que es fundamental para inducir la erección, y no cuestiona su relevancia para modificar la patología más allá de una vaga referencia a una mayor y más sostenida afluencia de sangre en los cuerpos cavernosos .

El nuevo mecanismo propuesto experimentalmente parte de la base que la ED, como todo desorden funcional, surge de un daño subyacente en los tejidos responsables de la función respectiva, y que para realmente tratar la ED en forma curativa y no meramente paliativa es necesario contrarrestar y/o reparar ese daño, no simplemente inducir una erección**.** En el caso de la ED vasculogénica, los tejidos afectados son el endotelio y la musculatura lisa, tanto en la región trabecular de los cuerpos cavernosos como en la media de las arterias que los irrigan **(7,19);** y, en el caso de la neuropraxia experimental, preceden temporalmente al daño funcional **(46).** Aunque la disfunción endotelial juega algún papel en ED, ésta dista de tener la significación que tradicionalmente se le asigna en el sistema vascular sistémico (extrapeneano). Ello se debe a que, primero, no existe una demostración fehaciente de un papel protagonista de la disfunción endotelial (aunque obviamente existe) en la ED; y, segundo, porque en estas estructuras este papel fundamental lo desempeña la musculatura lisa trabecular, cuya dilatación por el NO/cGMP lleva a la erección.

Si la musculatura lisa corporal no se dilata adecuadamente durante el estimulo sexual o farmacológico, que lleva a la producción requerida de NO/cGMP, las venas no se comprimen pasivamente contra la túnica albugínea reteniendo la sangre en los cuerpos cavernosos y se produce la "fuga venosa" ("venous leakage"), otra forma de denominar la CVOD. Ello se demuestra experimentalmente en la rata por cavernosometría y cavernosografía **(57).** Si un defecto similar ocurre en

la capa media de la pared de las arterias del pene, hay una insuficiencia de irrigación, o ED arteriogénica; y si ambos procesos coexisten la ED vasculogénica es mixta. Es importante enfatizar que la CVOD es la forma primordial, seguida por la mixta, mientras que la contribución de la insuficiencia arterial a la ED vasculogénica es debatible. Pero en todos estos casos es la "rigidez" de la musculatura lisa la que causa la incapacidad de los cuerpos cavernosos y las arterias del pene de dilatarse; la que impide paradójicamente alcanzar la rigidez del pene en la erección; y, a menudo, la responsable de la resistencia a los PDE5i orales "a demanda", e incluso a la inyección intracavernosa de substancias vasoactivas.

Una serie de estudios experimentales en modelos animales (ratas, ratones, conejos), particularmente en el ultimo quinquenio, ha permitido concluir que la rigidez progresiva de la musculatura lisa producida por los factores de riesgo para ED tales como envejecimiento, diabetes, hipertensión, y otras enfermedades vasculares, tiene un común denominador. Este es **la pérdida gradual de células musculares lisas** debido a apoptosis y a la disminución de la replicación celular que normalmente contrarrestaría la pérdida de estas células, y adicionalmente su reemplazo por fibras colágenas y otros componentes de la matriz extracelular, o sea una fibrosis difusa de los cuerpos cavernosos **(6)** La histopatología molecular y celular de esta condición tiene ciertas características muy similares a los de la placa de PD**,** involucrando la transformación fenotípica de fibroblastos en miofibroblastos y/o de células musculares lisas que pasan de la forma contráctil a la patológica, con depósito exagerado de colágeno**.**

El proceso condiciona una "atrofia" progresiva de la musculatura lisa; o, más concretamente, "**distrofia**", ya que va asociado con fibrosis y en ciertos casos aparición de adipocitos con depósitos grasos en los cuerpos cavernosos. Una extensión natural de estos estudios en animales experimentales fue demostrar que esta distrofia y la CVOD resultante **(26),** ocurre con características muy similares en una condición tradicionalmente considerada como ED neurogénica. Esta es precisamente la ED que se manifiesta en un considerable numero de pacientes a consecuencia de la prostatectomía radical por cáncer de próstata. Ello se ha observado en muestras de cuerpos cavernosos humanos **(44,45),** pero esencialmente en ratas donde se efectúa la resección de un pequeño trozo de los dos nervios cavernosos ("bilateral cavernosal nerve resection" o **"BCNR")** o uno solo ("unilateral CNR", o **"UCNR")** **(20,46,52,53);** o donde el daño a los nervios es menor, con contusión ("crush") controlada **(3,4,46).** Es bien claro que, tal como ocurre en la musculatura esquelética, el daño neural o neuropraxia funcional induce distrofia en la musculatura lisa con fibrosis, stress oxidativo progresivo y la aparición de CVOD; igual que en los modelos de envejecimiento, diabetes o isquemia. Ello explica porque muchos pacientes después de la prostatectomía radical desarrollan CVOD y esta es refractaria no ya solamente a PDE5i orales (en parte debido a la combinación con ED neurogénica) sino a la inyección directa de vasodilatadores en los cuerpos cavernosos (CVOD, o incluso ED vasculogénica mixta).

5.- Evidencia experimental del efecto beneficioso de la administración prolongada y continua de los PDE5i sobre la fisiopatología del cuerpo cavernoso y la CVOD, como enfoque "curativo".

El resultado de esta fibrosis gradual de los cuerpos cavernosos es la pérdida de relajación tisular y la resultante una CVOD que se detecta por cavernosometría de infusión dinámica en los modelos animales de factores de riesgo para ED, tales como daño a los nervios cavernosos, diabetes o envejecimiento **(20,26,2746,52,53,57,58).** Inspirado en el mecanismo endógeno de defensa por producción de iNOS que se observa en la placa de PD y en los cuerpos cavernosos durante el envejecimiento y la diabetes **(18),** se buscó elevar en forma continua y sostenida los niveles de NO y cGMP, mediante una administración oral semejante de generadores de óxido nítrico y PDE5i, tal como se había investigado en los modelos de PD. El periodo de administración en estos estudios, 45 días, fue equivalente a 3 % del lapso de vida de la rata, lo que trasladado al hombre, directamente y simplificando, sin considerar las diferencias de especies, podría ser equivalente a 2-3 años.

Basados en estos resultados se repitieron paradigmas similares en modelos de BCNR y UCNR, usando en tres estudios separados sildenafil, vardenafil y tadalafil en forma oral **(5,20,52,53)**. En todos los casos se llego a las mismas conclusiones: preservación en los cuerpos cavernosos del número de células musculares lisas, disminución de la muerte celular y la fibrosis, y prevención de la CVOD.

Las dosis diarias administradas en el agua de beber y en un caso en dos dosis diarias sublinguales, corregidas por las diferencias de especies para tener una idea aproximada en el hombre fueron respectivamente:

a) sildenafil aproximadamente 150 mg (75 mg en un nuevo estudio: **59**),

b) vardenafil, aproximadamente 40 mg, y

c) tadalafil, aproximadamente 30 mg,

Pero esta extrapolación requiere confirmación, ya que no se hicieron estudios farmacodinámicos. El periodo de aplicación debe ser lo más rápido posible tras de la neuropraxia para prevenir o revertir el rápido proceso de apoptosis **(60).** Vale la pena mencionar que en un estudio reciente se observo que una administración parecida de sildenafil a ratas con infarto de miocardio redujo el área fibrótica de la zona infartada y estimuló el funcionamiento cardiaco **(61),** reproduciendo estudios similares de otros grupos en ratones y que han estimulado o racionalizado los resultados clínicos en insuficiencia cardiaca **(62).**

Los estudios experimentales citados son alicientes, y lo mismo ocurre con estudios clínicos preliminares recientes, que sugieren que el tratamiento prolongado y continuo favorece la rehabilitación del pene**.** El tadalafilo podría ser el más adecuado por su larga vida media y la posibilidad de administrarlo 2 veces por semana, pero no por ello puede concluirse que sería superior a sildenafil o vardenafil. Sin embargo, es necesario en las futuras pruebas clínicas de rehabilitación del pene tras la

prostatectomía radical que se consideren las diferentes variables que pueden afectar los resultados y que los estudios animales no pueden resolver:

a) lapso de administración tras la prostatectomía radical;

b) agente seleccionado, dosis y frecuencia;

c) duración del tratamiento;

d) posibles efectos colaterales;

e) prueba de discontinuación prolongada ("wash-out);

f) posibilidad de continuar por un t iempo con una administración adicional esporádica y "a demanda" para facilitar el acto sexual.

Quizás uno de los aspectos más difíciles de incluir en estudios clínicos es la prolongación del tratamiento continuo (diario o 2-3 veces/semana) por más de 6 meses o un año, que los estudios animales y consideraciones básicas de fisiopatología exigen para una reversión de la fibrosis y la preconstitución de la relación musculatura lisa/colágeno. Por supuesto los estudios convincentes deben ser doble ciego, randomizados, controlados por placebo, multicentro, con un numero adecuado de pacientes, y preferentemente no basados solamente en cuestionarios tipo IIEF, sino en mediciones de la capacidad eréctil. La mayoría, sino todos, los estudios clínicos en curso con PDE5i tras prostatectomía radical usan sólo la administración "a demanda" para posibilitar el acto sexual, por lo que no deben confundirse con los de administración continua y sostenida por largo tiempo con enfoque "curativo" o "preventivo".

Sin embargo, la ED vasculogénica es una patología compleja y multifactorial que hace difícil que una sola intervención farmacológica pueda resolver el problema cuando se aborda un enfoque "curativo" y no paliativo como el estándar hasta el momento **(62).** Lo que si puede preverse es que nuevas investigaciones de laboratorio se enfocarán en disecar las distintas etapas de una distrofia progresiva de los cuerpos cavernosos, abordando en combinación distintos agentes farmacológicos, que restauren el equilibrio celular/extracelular. Por supuesto, en el caso de la ED producida por la prostatectomía radical, la reparación con **neurofilinas** de los neurofilamentos dañados **(50,51)** puede ser un elemento básico en esa combinación, que a través de acciones neurotróficas no sólo restauren la conducción nerviosa dañada, sino estimulen la reparación de la musculatura lisa en una reversión de la atrofia inicial. Por ultimo, es importante tener presente el creciente interés en la posibilidad de que la administración crónica de PDE5i pueda ser útil para el tratamiento de la sintomatología del tracto inferior urinario ("lower urinary tract symptoms", o "LUTS") **(63,64)**, la ya discutida PD **(9),** o incluso condiciones no urológicas **(65),** basado en los mismos conceptos.

Aún cuando el traslado a la clínica es más remoto, es probable que la implantación intracavernosa de **células madre** para reemplazar las células musculares lisas perdidas o defectuosas en los cuerpos cavernosos, constituya una nueva terapia para CVOD. Ello particularmente después de prostatectomía

radical, tal como se ha demostrado extensamente para restaurar la musculatura lisa en los cuerpos cavernosos en distintos modelos de ED **(66,67)**, o incluso de reparación de la vagina **(68)**, y usando varios tipos de células madre. Más aun, la combinación de implantación de células madre con la administración continua y a largo plazo de PDE5i está siendo estudiada **(61),** basada en la capacidad del óxido nítrico y el cGMP de estimular la diferenciación de células madre en los linajes celulares deseados, bajo la modulación paracrina o yuxtacrina concurrente del tejido receptor, en este caso la musculatura lisa, el endotelio, y los axones de los nervios cavernosos**.**

6.- Resumen.

Muchos laboratorios de investigación básica continúan investigando y proponiendo enfoques terapéuticos novedosos para la Enfermedad de La Peyronie y la Disfunción Eréctil, basados en conceptos desarrollados en la última década sobre un común denominador histo-fisiopatológico: **la fibrosis tisular progresiva**. En la PD este proceso afecta la túnica albugínea, es relativamente rápido y lleva a una o varias placas fibróticas localizadas; En cambio, en el tipo más común de ED, la disfunción córporo-veno-oclusiva, es generalmente lento, difuso, y afecta la musculatura lisa de los cuerpos cavernosos.

Los estudios de tratamientos con más inmediato potencial clínico, basados en las citadas pruebas en modelos animales, apuntan a la administración sostenida y a largo plazo de PDE5i, particularmente en la etapa inicial de PD; pero se requieren ensayos clínicos controlados. Sin embargo, estos agentes per se podrían no ser suficientemente efectivos en el hombre, y quizás convendría investigar regimenes de combinación con otros fármacos que, como la pirfenidona o la pioglitazona, están ya en el armamentario clínico. De todos los potenciales enfoques experimentales que aun no han sido investigados en pruebas preclínicas específicamente para PD, quizás el más prometedor es la selección de una MMP en función de la expresión de colágenos y de la relación MMP/TIMP para potenciar la degradación selectiva de la placa.

Los modelos de disfunción eréctil tales como envejecimiento, diabetes, hipertensión, y otras enfermedades vasculares, tiene un común denominador. Este es **la pérdida gradual de células musculares lisas** debido a apoptosis y a la disminución de la replicación celular; y su reemplazo por fibras colágenas y otros componentes de la matriz extracelular, o sea una fibrosis difusa de los cuerpos cavernosos. La histopatología molecular y celular de esta condición tiene ciertas características muy similares a los de la placa de PD**,** involucrando la transformación fenotípica de fibroblastos en miofibroblastos y/o de células musculares lisas que pasan de la forma contráctil a la patológica, con depósito exagerado de colágeno**.**

Los resultados con modelos experimentales animales de lesión uni o bilateral de los nervios erectores con tratamiento inmediato de inhibidores de fosfodiesterasa 5, usando en tres estudios separados sildenafil, vardenafil y tadalafil en forma oral han llegado en todos los casos a las mismas conclusiones:

preservación en los cuerpos cavernosos del número de células musculares lisas, disminución de la muerte celular y la fibrosis, y prevención de la CVOD.

La rehabilitación del pene farmacológica que actualmente se considera en la clínica se basa en el tratamiento en forma crónica y continua, desde el momento más temprano posible tras la intervención quirúrgica, con PDE5i para la disfunción vasculogénica y con agentes neuromoduladores (neurofilinas) que promuevan la regeneración de los axones neuronales dañados por la cirugía**.**

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Abreviaturas

AGE: productos de glicosilación avanzada Terminal; **cGMP:** GMP cíclico; **CVOD:** disfunción veno-oclusiva corporal**; ED:** disfunción eréctil**; iNOS (NOS-II)**: sintetasa inducible del óxido nítrico; **MMPS**: metaloproteinasa; **nNOS (NOS I)**: NOS neuronal; **PAI-1:** inhibidor 1 del activador de plasminógeno **PD:** enfermedad de La Peyronie; **PDE5i:** inhibidores de fosfodiesterasa 5; **ROS:** especies de oxigeno reactivo; **TGFβ1:** factor de transformación y crecimiento celular beta 1; **TIMP:** inhibidor tisular de MMP

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AMELIORATION OF DIABETES-INDUCED FIBROSIS BY ANTIOXIDANT AND ANTI-TGFβ1 THERAPIES IN THE PENILE CORPORA CAVERNOSA IN THE ABSENCE OF iNOS EXPRESSION

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Running Title: Corporal fibrosis in the diabetic iNOS knockout mouse

Key words: erectile dysfunction, smooth muscle, penis, collagen, nitric oxide, decorin, allopurinol, molsidomine, apoptosis

Abbreviations: ASMA: α-smooth muscle actin; **CVOD:** corporal veno-occlusive dysfunction; **ED**: erectile dysfunction; **iNOS:** NOS II, inducible nitric oxide synthase; **iNOSKo:** iNOS knockout mouse; **PCNA**: proliferating cell nuclear antigen; **QIA:** quantitative image analysis**; SMC:** smooth muscle cells; **XOR:** xanthine oxidoreductase.

ABSTRACT

Erectile dysfunction **(ED)** associated with diabetes usually presents as corporal veno-occlusive dysfunction **(CVOD)**, which results from an impaired corporal tissue compliance caused by a loss of smooth muscle cells **(SMC)** and an increase in fibrosis. The observed expression of inducible nitric oxide synthase **(iNOS)** counteracts these processes through the steady production of nitric oxide and cGMP that inhibit TGFβ1 expression, oxidative stress, and collagen deposition. This mechanism is particularly evident in the iNOSKo mouse, where iNOS depletion reduces the corporal SMC/collagen ratio and SMC content, and increases collagen and oxidative stress, processes that are exacerbated by streptozotocin-induced diabetes and counteracted by insulin. We have now investigated whether sustained long-term separate treatments of diabetic iNOSKo mice with allopurinol, an antioxidant inhibiting xanthine oxidoreductase **(XOR)**, decorin, a TGFβ1-binding antagonist, and molsidomine, a long-life nitric oxide donor, prevent these processes. Eight-week treatments with allopurinol or decorin counteracted the decrease in SMC evidenced by α-smooth muscle actin **(ASMA)** and cell replication indicated by proliferating cell nuclear antigen **(PCNA),** and the increases in apoptosis by TUNEL and local oxidative stress by XOR, observed in corporal tissue sections. Only decorin increased the SMC/collagen ratio, and systemic oxidative stress, assayed by GSH/GSSG in blood, was only prevented by allopurinol. Compensation of iNOS absence by molsidomine was effective in reducing local and systemic oxidative stress, but did not prevent fibrosis. In conclusion, both allopurinol and decorin appear as promising approaches in single or combined pharmacological modalities for protecting the corporal SMC, but their functional effects need to be defined.

INTRODUCTION

We have shown in human tissues and in animal models that the combination of fibrosis and oxidative stress, either localized or diffuse, is the common pathophysiological denominator of the two major disorders affecting the penis, namely Peyronie's disease **(1,2)** and the most common form of erectile dysfunction: corporal veno-occlusive dysfunction **(CVOD) (2),** despite their different etiologies. In the case of CVOD this occurs in conditions as varied as aging **(3,4),** types 1 and 2 diabetes **(5,6)**, cavernosal nerve damage **(7-10)** and hypertension **(11).** The combined production of active TGFβ1, reactive oxygen species **(ROS**), and other profibrotic factors, stimulates the excessive deposition of collagen and extracellular matrix **(ECM)** by fibroblasts and myofibroblasts in the tunica albuginea and corpora cavernosa. The corporal smooth muscle cells **(SMC)** also undergo a switch from the contractile phenotype to synthetic phenotype, leading to ECM deposition. In the specific case of CVOD this is compounded by a loss of SMC, with the resulting decrease in the SMC/collagen ratio that impairs the compliance of the corpora cavernosa to relaxation by the nitric oxide/cGMP pathway and the resulting passive occlusion of the veins against the tunica that in the rat is measured by cavernosometry **(12)**.

Another common denominator of both Peyronie's disease and CVOD is the steady expression of inducible nitric oxide synthase **(iNOS)** by different cell types leading to the sustained generation of nitric oxide and cGMP that inhibit myofibroblast generation or SMC activation and collagen synthesis **(12,13).** Nitric oxide also reduces the profibrotic effects of oxidative stress by quenching ROS, and also stimulates collagen degradation and protects the SMC, so iNOS induction is considered to act as an antifibrotic mechanism. This role is supported by the inhibition of oxidative stress and fibrosis by iNOS gene transfer or long-term continuous administration of nitric oxide generators and PDE5 inhibitors **(3,8-10,14,15)** or by the exacerbation of these processes by chronic inhibition of iNOS activity by L-NIL **(9,16,17).**

Moreover, the genetic inactivation of iNOS expression in the iNOSKo mouse leads per se to corporal fibrosis and oxidative stress and this is compounded by diabetes **(18).**

Experimental approaches to ameliorate the underlying fibrotic histopathology induced by diabetes and particularly the one derived from cavernosal nerve damage with PDE5 inhibitors **(19-21)** still need clinical validation. To increase their efficacy it may be necessary to combine agents targeting different fibrotic pathways. One of the most obvious is the use of long half-life nitric oxide generators to mimic the effects of iNOS induction, such as molsidomine or SIN-10, an agent currently studied clinically as a vasodilator for the treatment of coronary artery disease and angina pectoris **(22-24),** and experimentally for its antifibrotic effects in the kidney and liver **(25-27)**, but not investigated so far for counteracting penile corporal fibrosis.

A second type of agents is antioxidants more effective than vitamin E and similar by targeting xanthine oxidoreductase **(XOR),** a critical enzyme in oxidative stress in the penis, such as allopurinol, widely applied clinically **(28,29),** and having a potent experimental antifibrotic action not yet explored for ED **(30-32)**. Finally, agents that aim to inactivate TGFβ signaling, such as decorin, a proteoglycan endogenously expressed in many organs, that binds several members of the TGFβ super-family is being pre-clinically investigated as an antifibrotic agent in wound healing and kidney, heart, and skeletal muscle fibrosis **(33-38)**, but we are not aware of related publications for corporal fibrosis.

The streptozotocin-induced diabetic iNOSKo mouse model, where we studied the impact of diabetes on the corpora cavernosa under conditions of iNOS deprivation that exacerbates fibrosis **(18),** lends itself for the investigation of antifibrotic and antioxidant compounds with potential for the prevention or reversion of this process. In the current study we have tested in the diabetic iNOSKo mouse the effects of the continuous long-term separate administration of allopurinol, decorin, and molsidomine on corporal fibrosis, oxidative stress, and SMC turnover.

MATERIALS AND METHODS

Animals and treatments

All the experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at our institution, and according to the NIH Guide for the Care and Use of Laboratory Animals. Four months old iNOSKo B6.129P2-Nos2tm1Lau/J **(iNOSKo)** mice were divided into the following groups that were maintained for 8 weeks before sacrifice (n=8 mice/group): 1) iNOSKo injected once ip with 150 mg/kg BW streptozotocin **(iNOSKo+STZ)**; 2) as #1 treated with 40 mg/Kg/day allopurinol in the drinking water **(iNOSKo +STZ+ALLO)**; 3) as #1 treated with 50 μg decorin per animal; ip twice a day (4 mg/kg/day) **(iNOSKo +STZ+DECO)**; 4) as #1 treated with 5 mg/Kg BW molsidomine ip daily **(iNOSKo +STZ+MOL).**.

Body weights were recorded weekly. Blood for glycemia determination was withdrawn at baseline and then weekly under 3% isofloruane anesthesia. Urine was collected from the urinary bladder under anesthesia before sacrifice. Mice were euthanized by a bolus administration of sodium penthobarbital. Blood for the determination of the GSH/GGSG ratio was collected from the heart. Penises were rapidly excised, weighed, and the shaft was skin denuded, a mid region was fixed in 10% formalin for tissue sectioning and the rest was frozen onto dry ice and stored at -80C for further use.

Determinations in fresh tissue and blood.

Glycemia was determined in serum by an Accu-Chek Active blood glucose meter (Roche, Ireland), and urinary glucose, ketone bodies, specific gravity, pH, and protein were determined by Multistix Dip Stick (Bayer).

Collagen estimation in fresh tissue was as previously described **(14)**.*To be done by Dolores.* Briefly, the tissue was homogenized in saline, hydrolyzed with 2N NaOH for 30 min at 120 °C, followed by the measurement of hydroxyproline by a modification of the Neumann and Logan's reaction using Chloramine T and Ehrlich's reagent, against a hydroxyproline standard

curve and measuring at 550 nm. Values were expressed as µg of collagen per mg of tissue.

For the measurement of GSH/GSSG ratio **(16),** blood was collected with or without 1-methyl-2 vinylpyridinium trifluoromethane sulfonate (M2VP) scavenger of reduced glutathione, described in the commercial kit protocol ("Bioxytech GSH/GSSG-412 kit" from Oxis Health Products). The omission or addition of M2VP allows the measurement of reduced (GSH) and oxidized (GSSG) glutathione, respectively. The spectrophotometric detection was recorded at 412 nm for 3 min after the addition of 3.8 µmol NADPH. The GSH/GSSG ratio is inversely related to ROS levels.

Histochemistry and immunohistochemistry

Paraffin embedded tissue sections (5 um) were used for the following procedures **(3.4.7-10)**: a) Masson trichrome staining for collagen (blue) and SM cells (red)**;** b) immunodetection with: 1) monoclonal antibody against α-smooth muscle actin **(ASMA)** as a SM cell marker (Sigma kit, Sigma Diagnostics, St Louis, MO); 2) polyclonal antibody against TGF-β1 (1:200) (Promega, Madison, WI), as pro-fibrotic factor**;** 3) monoclonal antibody against proliferating cell nuclear antigen **(PCNA)** as marker of cell proliferation (1:400) (Chemicon, Temecula, CA); and d) polyclonal antibody against XOR (1:5000; Abcam), as a marker of oxidative stress**.** The specificity of the antibodies was validated by western blot.

Briefly, tissue sections were treated with proteinase K (20µg/ml), followed by quenching in 0.3% H_2O_2-PBS, blocked with goat serum (Vector Laboratories, Burlingame, CA), and incubated overnight at 4C with the primary antibody. In the case of PCNA and XOR, antigen retrieval was performed by boiling the slides for 3 minutes in an antigen unmasking solution (Vector Labs). After the overnight incubation with the first antibodies, sections were then incubated with biotinylated anti-Mouse IgG (ASMA, PCNA), or biotinylated anti-Rabbit IgG (TGF-β1, XOR), respectively, followed by ABC complex (Vector labs) and 3,3'diaminobenzidine (Sigma) (PCNA and iNOS), or with the ASMA Sigma kit (ASMA) and 3-amino-9-ethylcarbazole**.**

TUNEL assay was performed as described **(3,4,7-10)** by applying the Apoptag peroxidase detection assay (Chemicon), with TdT enzyme and anti-digoxigenin-conjugated peroxidase, and 3,3'diaminobenzidine/H2O2. Sections were counter-stained with hematoxylin QS (Vector labs). Negative controls in the immunohistochemical detections were done by replacing the first antibody with IgG isotype. The negative control for TUNEL was by substituting buffer for the TdT enzyme. Testicular tissue sections were used as positive control for TUNEL.

Quantitative image analysis

Quantitative image analysis **(QIA)** was performed by computerized densitometry using the ImagePro 4.01 program (Media Cybernetics, Silver Spring, MD), coupled to an Leica B microscope equipped with an Spot RT digital camera (Diagnostic Instruments, Portland OR) **(1-7).** For Masson staining, 40x magnification pictures of the whole penis were analyzed for SM cells (stained in red) and collagen (stained in blue), and expressed as SM/collagen ratio. For ASMA and XOR staining, only the corpora cavernosa were analyzed in a computerized grid and expressed as % of positive area vs. total area of the corpora cavernosa. For PCNA and TUNEL determinations, the number of positive cells at 400X was counted and results were expressed as a % of positive cells/total cells in the corpora cavernosa. In all cases, 4 penile anatomically matched tissue sections per animal were examined at 40x, with enough fields to cover the whole corpora cavernosa, and in certain cases at 400x and 8 fields per section, with 8 animals per group.

Statistical analysis

Values were expressed as mean ± SEM. The normality distribution of the data was established using the Wilk-Shapiro test, followed by one way analysis of variance (one way ANOVA), and post-hoc comparisons with the Bonferroni test, according to the GraphPad Prism V 4.1. The three WT groups were compared among themselves, and the same was done

independently for the three iNOSKo groups. When measurements were restricted to two WT and two iNOSKo groups, comparisons were done among all groups. In some instances, the three WT groups were compared among themselves, and the same was done independently for the three iNOSKo groups, as indicated in the figures. For collagen content, comparisons were made only against the control WT by a two-tailed t test. Differences were considered significant at $p < 0.05$.

RESULTS

The effects of iNOS deletion and of diabetes induction in the streptozotocin-injected iNOSKo mice have been described previously **(18),** showing a reduction in the corporal SMC/collagen ratio and the SMC content in the iNOS ko mice as compared to the WT mice. STZ-induced diabetes led to further reduction, that was completely prevented by insulin in the WT mice but only partially in the iNOS ko mice.

In the current work, as expected eight-week treatments of this model with allopurinol, decorin, and molsidomine, did not affect significantly body weight from the value of 25.0 ±1.5 g at sacrifice in the untreated control animals. Similarly, allopurinol and decorin did not affect significantly the streptozotocin-induced hyperglycemia, but surprisingly molsidomine increased it to 431+/-32 from 354+/-17 mg/dl in the untreated control (p<0.001), and induced a considerable glucosuria from a basal level in the control (not shown). No ketonuria was found in any case, but the considerable proteinuriain the control (72.5±33.6 mg/dl) was significantly reduced by allopurinol to 15.4+/-5.5 mg/dl, suggesting a protective effect directly on the kidney tissue independent from glycemic control. Decorin and molsidomine did not affect proteinuria. As expected, nitrites in urine were present in all molsidomine treated animals

The effects of these treatments in preventing the underlying histopathology caused by diabetes and iNOS deletion in the corpora cavernosa of the iNOSKo mice were determined in

paraffin-embedded corporal tissue sections. **Fig. 1,** like the other figures, shows representative pictures for each group and bar graphs for the quantitative image analysis. Despite our initial assumption, neither allopurinol nor molsidomine affected the SMC/collagen ratio estimated by Masson trichrome. However, decorin did increase it considerably, by 2.5 fold. In contrast, the SMC content **(Fig. 2),** as estimated by ASMA, was more sensitive since not only decorin increased by 2.0-fold but also allopurinol by 2.3-fold. Although molsidomine exerted a smaller stimulation, it did not achieve statistical significance.

The protective effects of both decorin and allopurinol on the corporal SMC were reflected in significant reduction of the the apoptotic index **(Fig. 3),** but molsidomine did not reduce cell death. **(Fig. 4 A).** Allopurinol increased cell replication by 1.5 fold whereas decorin was ineffective **(A),** but the positive cell turnovers (proliferation predominating over cell death) were increased in both cases **(B).**

> **Comment:** I will not comment on molsidomine since it does not change the cell turnover. It is possible that the high replication try to compensate for high cell death

The three types of treatments were uniformly effective in reducing oxidative stress in the corpora cavernosa by 46-60% as estimated by XOR **(Fig. 5A).** This is reflected in the expected decrease of systemic oxidative stress by allopurinol and molsidomine, represented by the nearly 3-fold decrease of ROS in the blood, measured by the GSH/GSSG ratio (the higher, the lower the oxidative stress) **(B)**. However, and also as we expected since decorin acts by a different mechanism than antioxidant or nitric oxide release effects, this agent did not reduce systemic oxidative stress.

Finally, none of the protective effects of these agents on the corporal histology seemed to be due to reducing the expression of the key pro-fibrotic factor TGFβ1, and even in one case, allopurinol, this expression was surprisingly stimulated by 2.0-fold (not shown**).** The fact that decorin did not induce any significant change is expected from its mechanism of action based on its binding to TGFβ1, and thus neutralizing it, without affecting its expression.

DISCUSSION

This report is the first to compare concurrently in a mouse model of exacerbated fibrosis, the diabetic iNOSko mouse, the potential antifibrotic, antioxidant and SMC protective action of three pharmacological agents. They act by different mechanisms.on the penile corpora cavernosa, but coincide in reducing the levels of some but not all key pro-fibrotic factors, in one case ROS and the subsequent oxidative stress through direct antioxidant (allopurinol) or indirect quenching (molsidomine) processes, and in the other case of TGF β1 by binding it (decorin) and blocking the signaling triggered by its receptor. However, although decorin and allopurinol are effective in protecting the SMC through inhibition of apoptosis, unexpectedly molsidomine (which theoretically should replace the effects of the absent iNOS) was not.

The critical profibrotic role of TGFβ1 expression in the diabetic iNOSKo was confirmed by the results of a long-term treatment with decorin, a small leucine-rich proteoglycan that counteracts TGFβ1 binding to its receptor and thus acts as an antifibrotic agent **(33-38).** The increase in the corporal SMC/collagen ratio and SMC content while decreasing apoptosis, and the local tissue protection against oxidative stress without affecting systemic ROS, is in agreement with this mechanism. The effects of decorin have not been reported for penile tissues, other than in terms of the potentially compensatory expression of decorin observed in the human Peyronie's disease fibrotic plaque **(39).** However, our results do not predict how decorin would act in a setting of a normal iNOS induction, since TGFβ1 overexpression was not observed in the non-diabetic iNOSKo or in the diabetic wild type mice in comparison with the non-diabetic wild type animals **(18).** So far the role of TGFβ1 in corporal fibrosis induced by aging, diabetes, or cavernosal damage remains elusive, in contrast to its very clear significance for Peyronie's disease **(1,2),** and fibrosis of other organs such as the kidney, liver, and heart **(33-38).**

Allopurinol is perhaps the most promising agent since it was very effective in preventing corporal SMC loss in the diabetic iNOSKo by reducing apoptosis and oxidative stress, both systemic and local, and stimulating cell proliferation, thus confirming the beneficial effects of

antioxidant therapy on corporal fibrosis and ED in diabetes and on tissue fibrosis in general **(40-42).** The lack of allopurinol effects on the corporal SMC/collagen ratio, that disagrees with its well known reduction of collagen deposition in tissues such as the heart and liver **(30-32**), is probably related to the also unexpected increase in TGFβ1 expression. The systemic effects of allopurinol on ROS agree with the decrease in proteinuria that reflects an antifibrotic effect on diabetic nephropathy, previously reported for this agent, since allopurinol has been proved to be an effective antifibrotic and antioxidant in the heart and kidney **(30-32).** This also confirms the pivotal role of XOR in corporal oxidative stress that has been essentially ignored in favor of NADPH oxidase **(43,44)**, reflected on the absence of published reports on the effects of allopurinol on erectile dysfunction or the penis. The single exception is a study that showed acute effects of allopurinol in the attenuation of ischaemia- and reperfusion-induced corporeal injury in a rat model of veno-occlusive priapism, presumably based on the reduction of corporal lipid peroxidation **(45).**

Perhaps the most surprising result was the inability of the long-term administration of the long half-life exogenous nitric oxide generator, molsidomine, in affecting the SM/collagen ratio, SM content or apoptosis, that was expected from the antifibrotic effects reported for the kidney and liver **(25-27)**. Linked to the vasodilator activity but still poor efficacy of the acute administration of molsidomine or its derivative SIN-1 to induce corporal relaxation in comparison with PGE1 **(46,47)**, this may rule out further consideration of this drug for ED. However, its antioxidant activity, both local and systemic, supports the view that iNOS is in fact antioxidant through a sustained production of nitric oxide in the corpora cavernosa. Nitric oxide leads to XOR down regulation in addition to ROS quenching, and is not necessarily a cause of oxidative stress as it may occur in other tissue settings**.** In turn, the stimulation of cell proliferation by molsidomine in the corporal tissue by nitric oxide/cGMP agrees with our previous studies **(7,9,16),** and again establishes an interesting divergence with the effects in general seen in the

arterial wall SM **(48).** Therefore further studies may be needed to rule out any use for molsidomine for ED.

In summary, under conditions where the inactivation of the iNOS gene exacerbates corporal fibrosis in diabetes this histopathology is ameliorated by long-term pharmacological reduction of oxidative stress or counteracting TGFβ1, but not by simply producing nitric oxide from exogenous sources. This indicates that the use of certain antioxidant or antifibrotic agents would be effective to ameliorate corporal fibrosis and improve erectile dysfunction in diabetes, thus suggesting that combination therapy with some of these types of compounds, perhaps together with long-term continuous treatment with PDE5 inhibitors may be beneficial by targeting different sites in the fibrosis pathways. Allopurinol, because of his long clinical use and its oral route of administration, is an interesting candidate in this respect, despite the negligible effect on corporal collagen deposition observed here. Decorin was more uniformly effective, but we are not aware of any clinical use so far. Further investigations in animal models are required to confirm preclinically these assumptions, particularly by measuring the penile erection response that was not studied in the current report.

ACKNOWLEDGEMENTS

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LEGENDS TO FIGURES

Figure 1. Long-term treatment of the diabetic iNOSKo mice with decorin increases the corporal SMC/collagen ratio. Top: representative pictures of Masson trichrome staining. iNOSKo+STZ; streptozotocin-injected iNOSKo mouse, untreated; iNOSKo+STZ+ALLO**:** iNOSKo+STZ treated with allopurinol. iNOSKo+STZ+DECO**:** iNOSKo+STZ treated with decorin. iNOSKo+STZ+MOL**:** iNOSKo+STZ treated with molsidomine. Bottom: quantitative image analysis for the SMC/collagen ratio expressed as means+/-SEM; ***: $p < 0.001$.

Figure 2. Long-term treatment of the diabetic iNOSKo mice with decorin or allopurinol, increases the corporal SMC content. Top: representative pictures of ASMA immunostaining; symbols as for Fig. 1. Bottom: quantitative image analysis for the SMC content expressed as means+/-SEM; ***: $p < 0.001$; *: $p < 0.05$.

Figure 3. Long-term treatment of the diabetic iNOSKo mice with decorin or allopurinol, reduces the corporal apoptotic index. Top: representative pictures of apoptosis by TUNEL immunostaining; symbols as for Fig. 1. Bottom: quantitative image analysis for the apoptotic index expressed as means+/-SEM; ***: $p < 0.001$; *: $p < 0.05$.

Figure 4. Long-term treatment of iNOSKo mice with allopurinol increases corporal cell replication and induces a positive cell turnover. Top: representative pictures of PCNA immunostaining; symbols as for Fig. 1. Bottom: quantitative image analysis for the number of PCNA positive cells and for cell turnover expressed as means+/-SEM; *: $p < 0.05$.

Figure 5.   Long-term treatment of iNOSKo mice with allopurinol, decorin and molsidomine reduces corporal oxidative stress and, except for decorin, decreases systemic oxidative stress. Top: representative pictures of XOR immunostaining; symbols as for Fig. 1. Bottom: quantitative image analysis for the area of XOR+ staining and for the GSH/GSSG ratio in blood expressed as means+/-SEM; ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$.
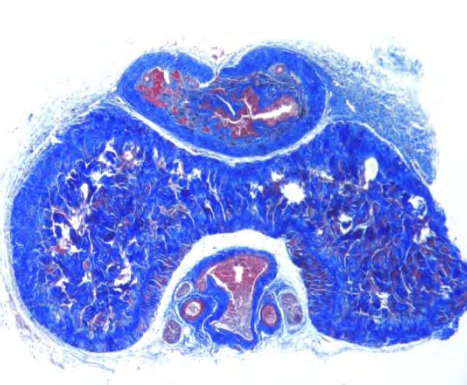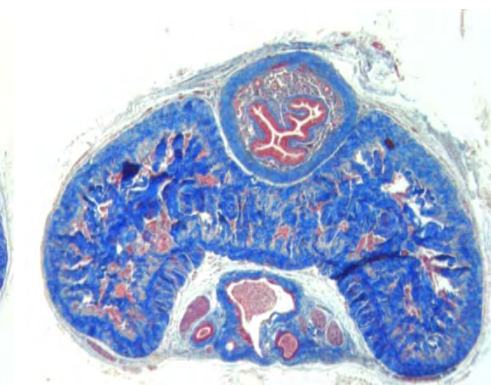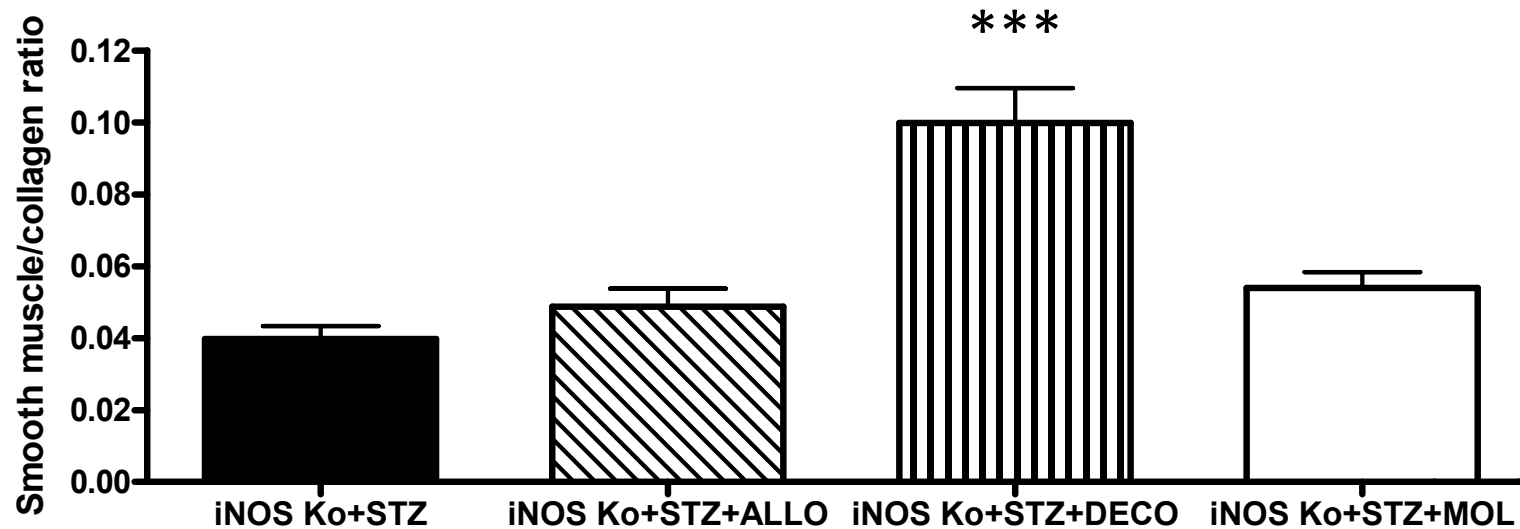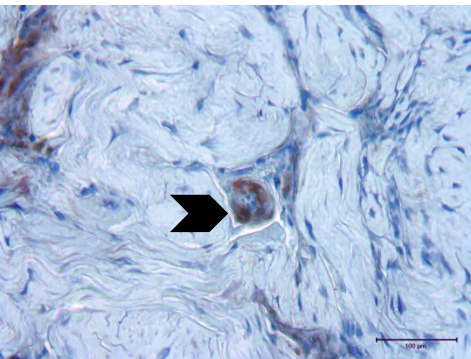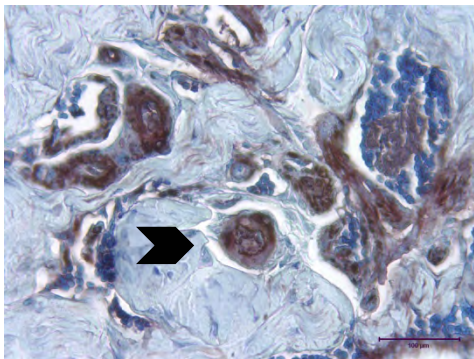
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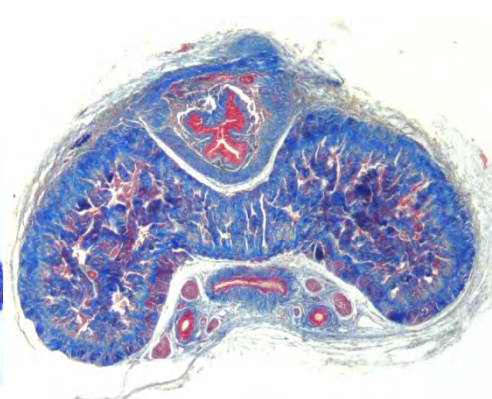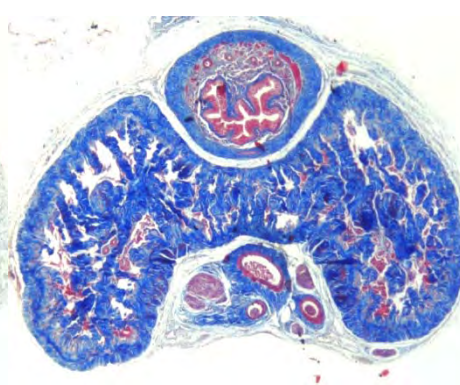
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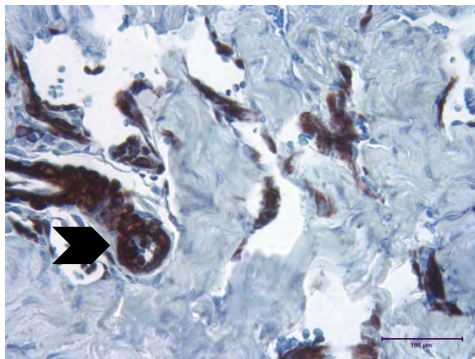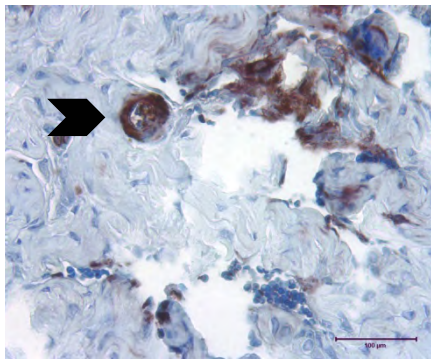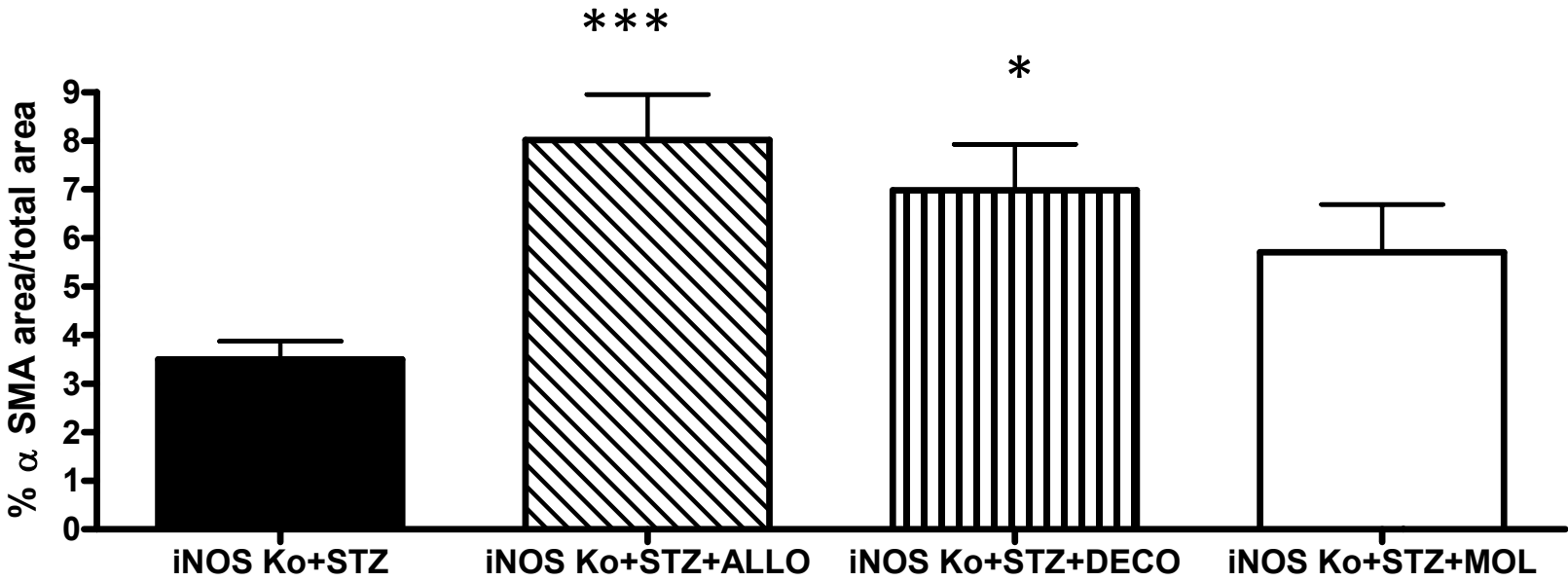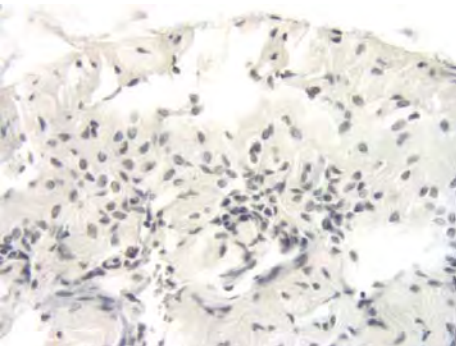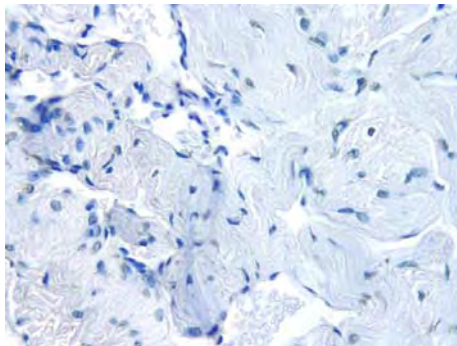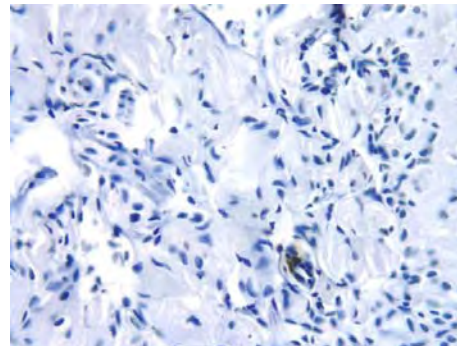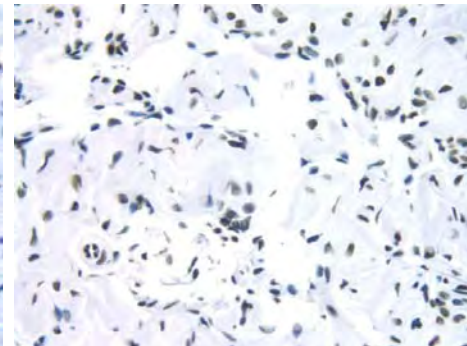
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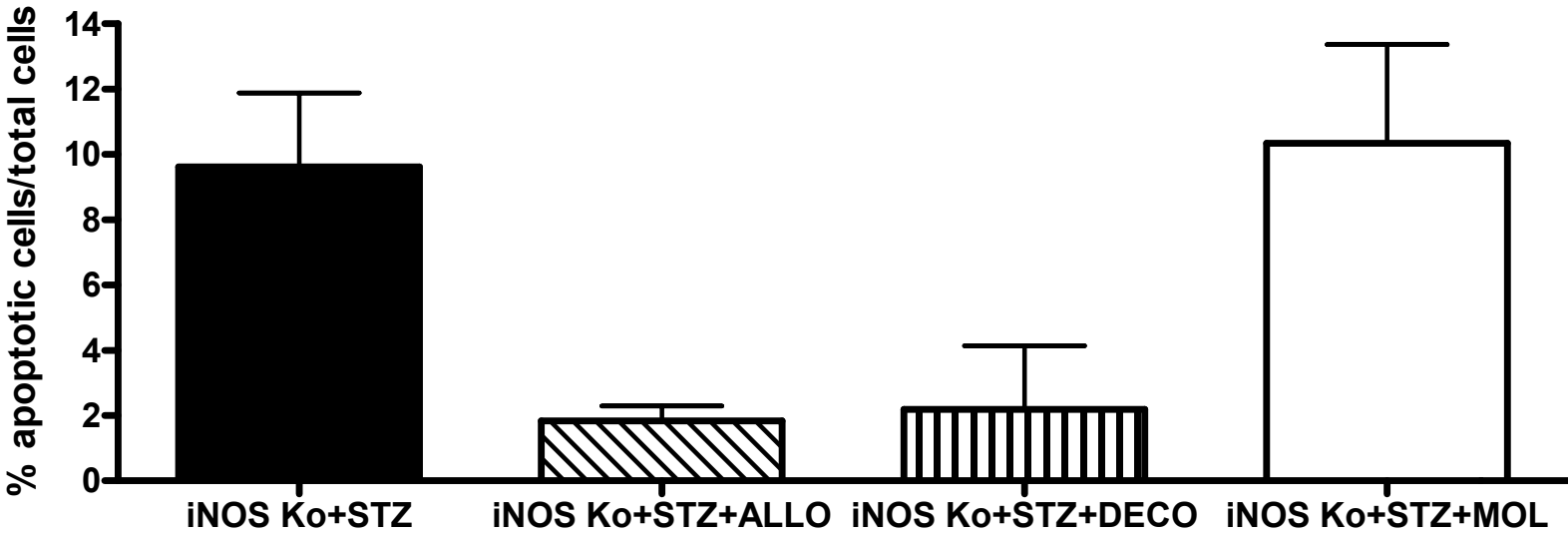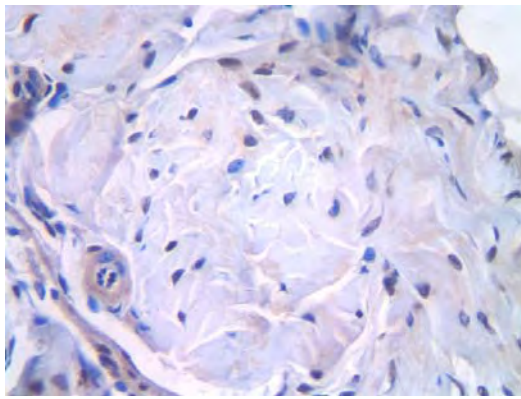
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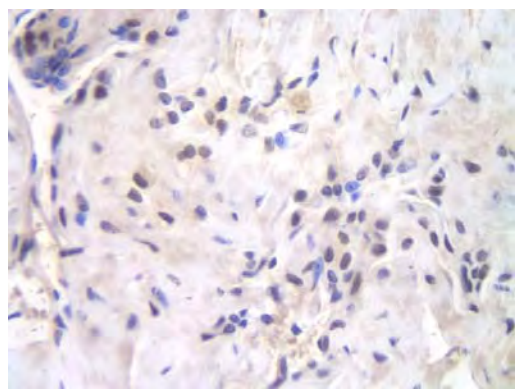
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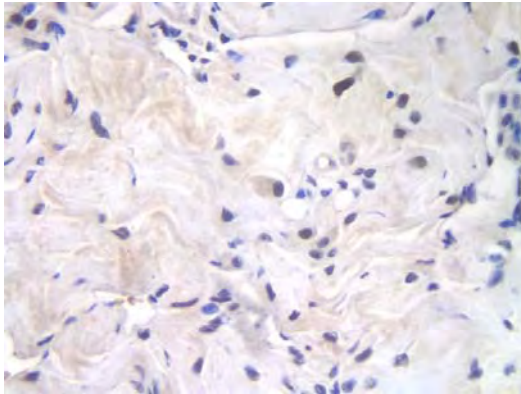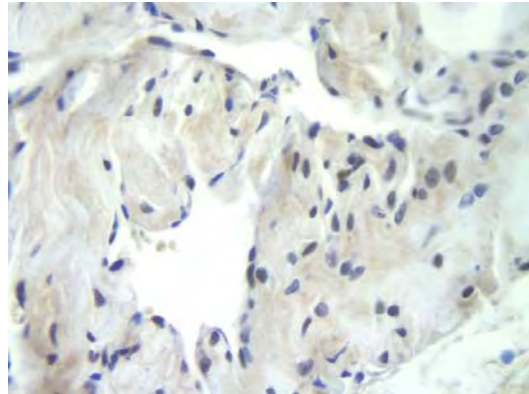
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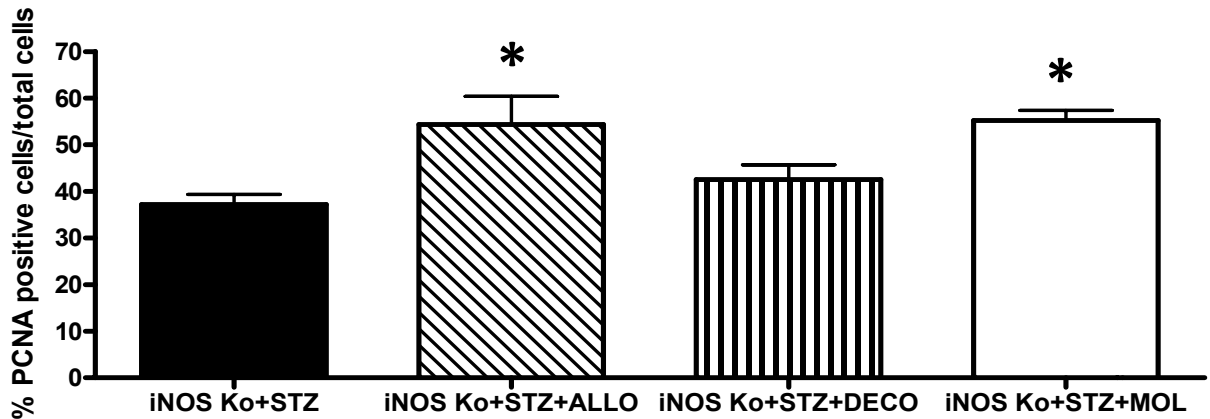
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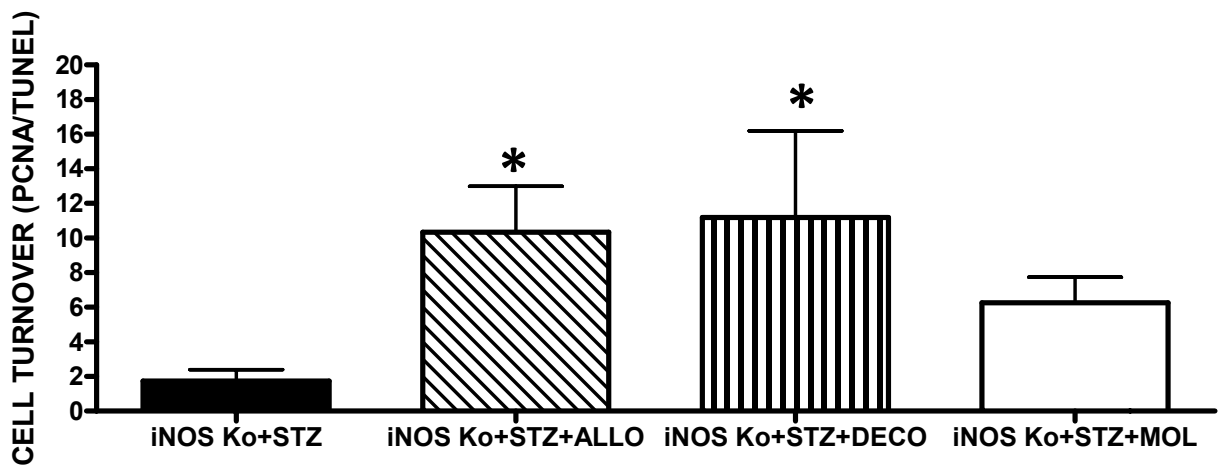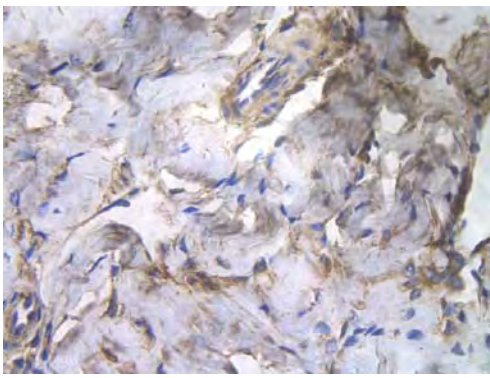
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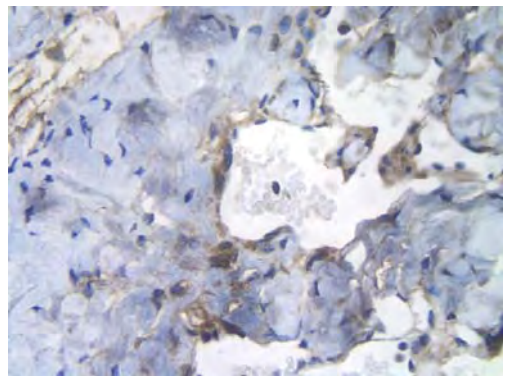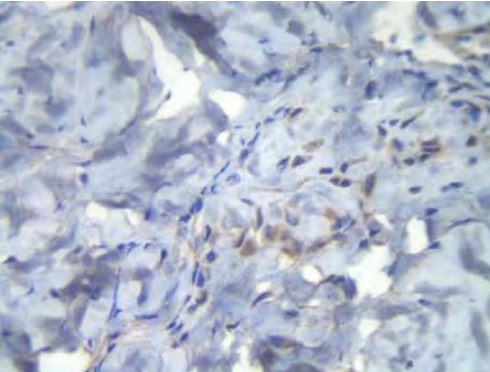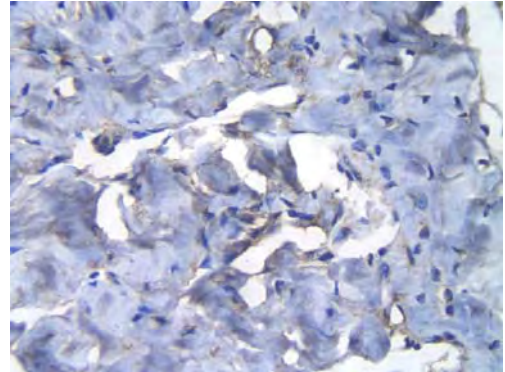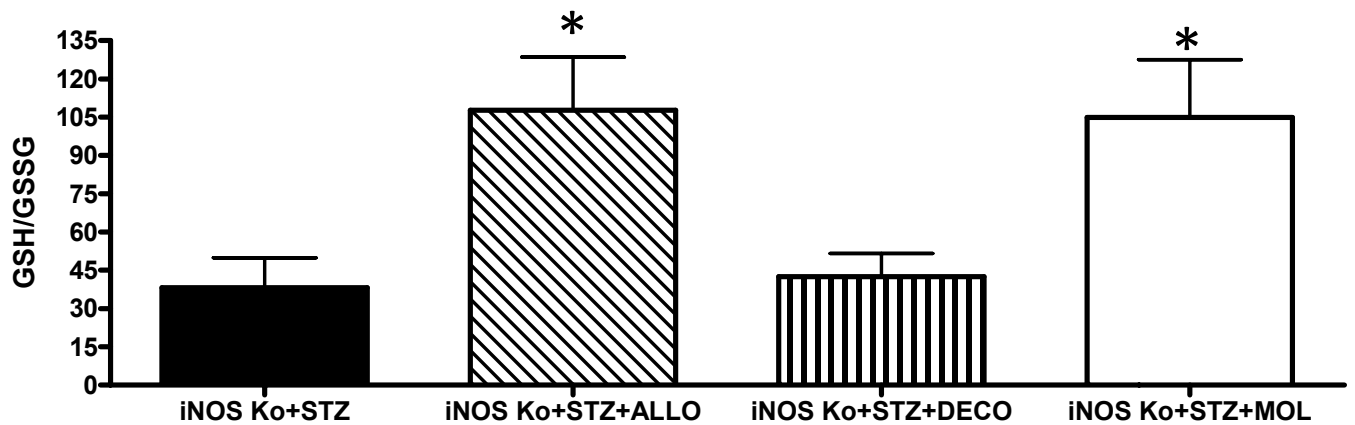
iNOSKo+STZ+DECO   iNOSKo+STZ+MOL

B

The Genetic Inactivation of Inducible Nitric Oxide Synthase (iNOS) Intensifies Fibrosis and Oxidative Stress in the Penile Corpora Cavernosa in Type 1 Diabetes

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ABSTRACT

Introduction. Endogenously elicited inducible nitric oxide synthase (iNOS) induction counteracts fibrosis and oxidative stress in penile tissues in rat models of Peyronie's disease and erectile dysfunction.
Aim. The current study aimed to determine whether the genetic blockade of iNOS expression in the iNOS knock out (iNOS KO) mouse intensifies fibrosis and oxidative stress in the penile corpora cavernosa, and this is exacerbated by streptozotocin (STZ)-induced diabetes and counteracted by insulin.
Main Outcomes Measures. Quantitative assessment of histological and biochemical markers in mouse corporal tissue.
Methods. Male iNOS KO and wild type (WT) mice were left untreated or injected with STZ, with or without insulin treatment. At 8 weeks, glycemia, glucosuria, and proteinuria were determined, and corporal tissue sections were obtained and subjected to Masson trichrome staining for smooth muscle (SM)/collagen ratio, and immunostaining for α-smooth muscle actin (ASMA) for, SM content, proliferating cell nuclear antigen (PCNA) for cell replication, TGFβ1 as profibrotic factor, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for apoptosis, and xanthine oxidoreductase (XOR) for oxidative stress. Collagen was estimated by the hydroxyproline reaction.
Results. The corporal SM/collagen ratio and SM content were reduced, and collagen content increased in iNOS KO mice as compared with WT mice, but apoptosis was decreased and cell replication increased, whereas TGFβ1 and XOR did not vary. Severe hyperglycemia caused in the WT a reduction of the corporal SM/collagen ratio and SM content and an increase in apoptosis without changes in PCNA, TGFβ1, or XOR. In the iNOS KO mouse the hyperglycemia-induced alterations were exacerbated, with additional increases in oxidative stress and TGFβ1. Insulin normalized glycemia and partially protected the SM in both the WT and the iNOS KO mice.
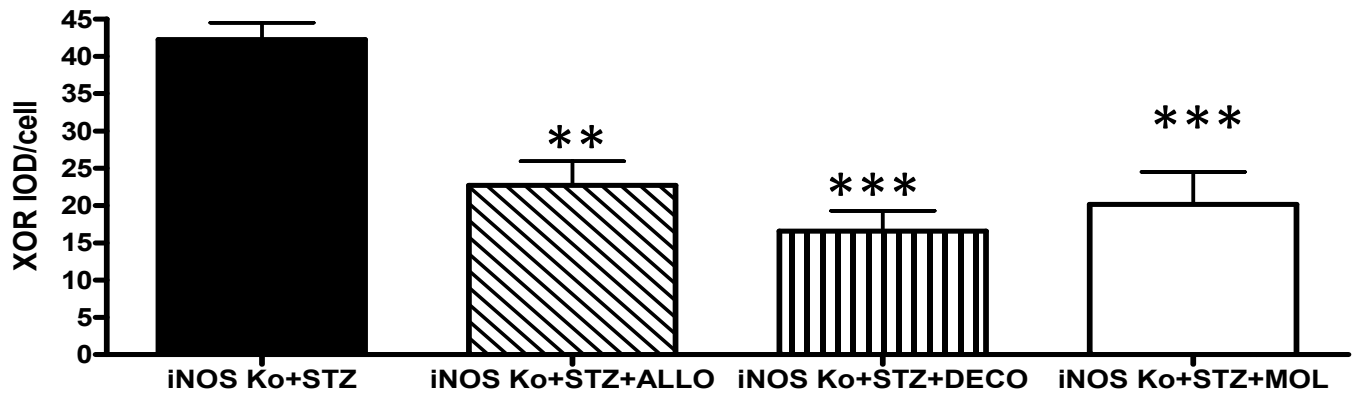*Conclusions.* The antifibrotic, antioxidative, and SM-protective roles of iNOS in the penile corpora cavernosa were confirmed in the iNOS KO/STZ mouse model. These findings support the importance of endogenously-elicited iNOS induction in protecting the penile corpora cavernosa from the pro-fibrotic effects of hyperglycemia. **Ferrini MG, Rivera S, Moon J, Vernet D, Rajfer J, and Gonzalez-Cadavid NF. The genetic inactivation of inducible nitric oxide synthase (iNOS) intensifies fibrosis and oxidative stress in the penile corpora cavernosa in type 1 diabetes. J Sex Med 2010;7:3033–3044.**

Key Words. Erectile Dysfunction; Smooth Muscle; Penis; Collagen; Nitric Oxide; Apoptosis

Introduction

Over the last few years it has become increasingly evident in men and in rat models that risk factors of erectile dysfunction (ED) such as aging, diabetes, hypogonadism, and nerve damage following radical prostatectomy promote smooth muscle (SM) dysfunction in the corpora cavernosa because of the loss or damage of SM, the tissue fibrosis, and the impairment of tissue relaxation that characterize vasculogenic, more specifically venogenic, ED [1–11]. The association between

the increase in collagen fibers and the reduction of SM cells by apoptosis, resulting in the decrease of the SM/collagen ratio, leads to a defective corporal compliance underlying the most prevalent form of ED, corporal veno-occlusive dysfunction (CVOD) [12]. Often, but not always, there is a parallel increase in the activation of the TGFβ1/Smad key pro-fibrotic pathway and of the production of reactive oxygen species (ROS) that cause oxidative stress and apoptosis and also stimulate fibrosis [7,13,14]. However, TGFβ1 expression and oxidative stress in the corpora cavernosa are less marked in CVOD than in the tunica albuginea in Peyronie's disease, or in other urological tissues such as in diabetic nephropathy [15–18].

These features in the corpora cavernosa, which are essentially an extension of the vascular system, are accompanied in the case of aging and diabetes by similar changes in the media of both the small penile arteries and large femoral artery and aorta vessels [19,20]. The fibrotic and oxidative stress processes are similar to those seen in Peyronie's disease [15,16], so that in this sense the "hardening" of all these tissues is the common denominator, which in the case of the corporal/vascular system is combined with the SM cell loss, leading to the subsequent functional impairment.

A remarkable constant process in this histopathology is the exacerbated expression of the inducible nitric oxide synthase (iNOS), a NOS isoform that is not expressed in normal penile tissues [21]. Three independent lines of evidence in rodent models support the view that in the penis, iNOS is an endogenous mechanism of defense against fibrosis and SM cell loss and not a deleterious factor contributing to SM dysfunction: (i) gene transfer of iNOS cDNA reduces fibrosis in the corpora and tunica [22,23] in a model of Peyronie's disease (PD); (ii) long-term specific inhibition of iNOS activity by L-NIL intensifies collagen deposition and fibrosis in the penis and arterial wall of old animals [19,24]; and (iii) the sustained pharmacological elevation of the levels of nitric oxide and/or its effector, cyclic guanosine monophosphate, mimicking the result of iNOS induction, also acts as an antifibrotic mechanism in an experimental model of aging and cavernosal nerve resection [1–5,25,26].

This "protective" iNOS induction has also been documented in tissues other than the penis or some arteries using the iNOS knock out mouse (iNOS KO). This strain served to show that in the rat kidney, the anti-inflammatory and antifibrotic effects of the type-1 cytokine response is iNOS dependent [27] and that iNOS deletion exacerbates interstitial fibrosis after unilateral ureteral obstruction [28], or in streptozotocin (STZ)-induced diabetes despite a compensatory production of endothelial NOS [29]. Similarly hepatic fibrosis in animals fed high-fat diet is exacerbated in the iNOS KO [30].

However, the postulation that iNOS is antifibrotic does not agree with other papers that do not show effects of iNOS deletion on fibrosis, such as in the heart after myocardial infarction [31] and in the liver after chronic carbon tetrachloride administration [32]. There are even reports claiming that it is the iNOS deficiency that provides protection against liver fibrosis in this model [32] and also in overload-induced cardiac hypertrophy [33] and in silica-induced pulmonary fibrosis [34]. It is possible that the deleterious effect of iNOS could be due in some cases to being produced as a result of a macrophage recruitment induced by the chemical insult instead of the progressive and milder metabolic changes caused by diabetes or aging.

Chronic inhibition of iNOS activity in wild type (WT) animals with L-NIL or aminoguanidine partially reduced proteinuria, an indicator of kidney fibrosis in 5/6 nephrectomized rats and ischemia/reperfusion injury [35,36]. In contrast, L-NIL increased myofibroblast accumulation in the "preconditioning" of the kidney to protect from ischemia/reperfusion, which agrees with the view that iNOS is essential for protecting the heart from ischemia/reperfusion [37,38]. This confusing evidence regarding the role of iNOS genetic deficiency in fibrosis of nonpenile tissues occurs also in terms of oxidative stress because some reports claim that this ablation is deleterious, and therefore that iNOS counteracts ROS generation, at least in heart and brain injury [39,40], whereas others postulate the opposite, for instance in myocardial infarction and atherosclerotic arteries [41,42].

As no studies are available on the histopathology of penile tissues in the iNOS KO, we decided to approach in this model the clarification of these controversies, focusing on the specific case of the penile corpora cavernosa subjected to the long-term effect of diabetes as a risk factor for ED. We have investigated whether the absence of iNOS in the iNOS KO and/or the development of STZ-induced diabetes neuropathy intensify SM loss, fibrosis, and oxidative stress in the penile corpora cavernosa. The results may help to clarify the role of iNOS in the development of fibrosis due to neuropathies and aging.

Methods

Animals and Treatments

All the experiments were approved by the Institutional Animal Care and Use Committee of LABioMed at Harbor-UCLA, Torrance, CA, USA, and according to the NIH Guide for the Care and Use of Laboratory Animals. Two-month old WT C57BL6J mice (WT) and iNOS KO B6.129P2-Nos2tm1Lau/J (iNOS KO) were divided into the following groups that were maintained for 8 weeks before sacrifice (N = 8 mice/ group): (i) WT, no treatment control (WT); (ii) WT injected with 150 mg/kg BW STZ to induce diabetes (WT + STZ); (iii) as #2, also injected daily ip with 0.05 IU/Kg BW insulin to exert glycemic control (WT + STZ + INS); (iv) iNOS KO, no treatment control (iNOS KO); (v) iNOS KO injected once ip with 150 mg/kg BW STZ (iNOS KO + STZ); (vi) as #5, also injected ip daily with 0.05 IU/Kg BW insulin (iNOS KO + STZ + INS). Insulin treatment started after the animals showed a glycemia >350 mg/dL.

Body weights were recorded weekly. Blood for biochemical determinations was withdrawn from the tail vein at baseline and then weekly under 3% isofloruane anesthesia. Urine was collected from the urinary bladder under anesthesia before sacrifice. Mice were euthanized by a bolus administration of sodium penthobarbital. The penises were rapidly excised and weighed, the shaft was skin denuded, a mid-region was fixed in 10% formalin for tissue sectioning, and the rest was frozen as fresh tissue at −80°C for other assays.

Determinations in Fresh Tissue and Blood

Glycemia was determined in serum by an Accu-Chek Active blood glucose meter (Roche, Ireland), and urinary glucose, ketone bodies, specific gravity, pH, and protein were determined by Multistix Dip Stick (Bayer, Pittsburgh, PA, USA).

Collagen estimation in fresh tissue was as previously described [24]. Briefly, the tissue was homogenized in saline, hydrolyzed with 2N NaOH for 30 minutes at 120°C, followed by the measurement of hydroxyproline by a modification of the Neumann and Logan's reaction using Chloramine T and Ehrlich's reagent, against a hydroxyproline standard curve and measuring at 550 nm. Values were expressed as μg of collagen per mg of tissue.

Histochemistry and Immunohistochemistry

Paraffin embedded tissue sections (5 um) were used for the following procedures [1–7]: (i) Masson trichrome staining for collagen (blue) and SM cells (red); (ii) immunodetection with (1) monoclonal antibody against α-smooth muscle actin (ASMA) as a SM cell marker (Sigma kit, Sigma Diagnostics, St Louis, MO); (2) polyclonal antibody against TGF-β1 (1:200) (Promega, Madison, WI, USA), as pro-fibrotic factor; (3) monoclonal antibody against proliferating cell nuclear antigen (PCNA) as marker of cell proliferation (1:400) (Chemicon, Temecula, CA, USA); and (iii) polyclonal antibody against xanthine oxidoreductase (XOR) (1:5000; Abcam), as a marker of oxidative stress. The specificity of the antibodies was validated by Western blot.

Briefly, tissue sections were treated with proteinase K (20 μg/mL), followed by quenching in 0.3% H_2O_2-PBS, blocked with goat serum (Vector Laboratories, Burlingame, CA, USA) and incubated overnight at 4°C with the primary antibody. In the case of PCNA and XOR, antigen retrieval was performed by boiling the slides for 3 minutes in an antigen unmasking solution (Vector Laboratories). After the overnight incubation with the first antibodies, sections were then incubated with biotinylated anti-Mouse IgG (ASMA, PCNA) or biotinylated anti-Rabbit IgG (TGF-β1, XOR), respectively, followed by ABC complex (Vector Laboratories) and 3,3'diaminobenzidine (Sigma) (PCNA and iNOS), or with the ASMA Sigma kit (ASMA) and 3-amino-9-ethylcarbazole.

TUNEL assay was performed as described [1–7] by applying the Apoptag peroxidase detection assay (Chemicon), with TdT enzyme and anti-digoxigenin-conjugated peroxidase, and 3,3'diaminobenzidine/H2O2. Sections were counter-stained with hematoxylin QS (Vector Laboratories). Negative controls in the immunohistochemical detections were done by replacing the first antibody with IgG isotype. The negative control for TUNEL was by substituting buffer for the TdT enzyme. Testicular tissue sections were used as positive control for TUNEL.

Quantitative Image Analysis (QIA)

QIA was performed by computerized densitometry using the ImagePro 4.01 program (Media Cybernetics, Silver Spring, MD, USA), coupled to an Leica B microscope equipped with an Spot RT digital camera (Diagnostic Instruments, Portland, OR, USA) [1–7]. For Masson staining, 40× magnification pictures of the whole penis were analyzed for SM cells (stained in red) and collagen (stained in blue) and expressed as SM/collagen ratio. For ASMA and XOR staining, only the
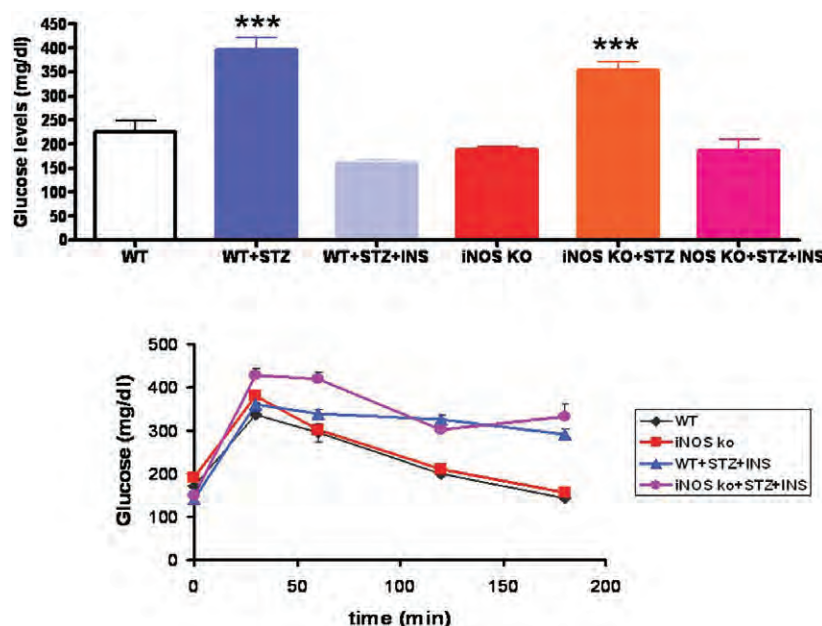
Figure 1 A single injection of streptozotocin (STZ) at a high dose elicits in the inducible nitric oxide synthase (iNOS) knockout (iNOS KO) mouse a level of hyperglycemia similar to the one in the wild type (WT) mouse, and this is controlled by long-term administration of insulin. Top panel: final values of nonfasting glycemia at 8 weeks after STZ injection, immediately before sacrifice. Bottom panel: oral glucose tolerance test carried out at 7 weeks. WT + STZ = WT treated with STZ; WT + STZ + INS = WT + STZ treated with insulin; iNOS KO + STZ = iNOS KO treated with STZ; iNOS KO + STZ + INS = iNOS KO + STZ treated with insulin. Values are means $\pm$ SEM. ***$P < 0.001$, for WT + STZ and iNOS KO + STZ vs. all others.

corpora cavernosa were analyzed in a computerized grid and expressed as % of positive area vs. total area of the corpora cavernosa. For PCNA and TUNEL determinations, the number of positive cells at 400× was counted and results were expressed as a % of positive cells/total cells in the corpora cavernosa. In all cases, four penile anatomically matched tissue sections per animal were examined at 40×, with enough fields to cover the whole corpora cavernosa, and in certain cases at 400× and eight fields per section, with eight animals per group.

Statistical Analysis

Values were expressed as mean $\pm$ SEM. The normality distribution of the data was established using the Wilk-Shapiro test, followed by one-way analysis of variance (ANOVA), and post-hoc comparisons with the Bonferroni test, according to the GraphPad Prism V 4.1. The three WT groups were compared among themselves, and the same was done independently for the three iNOS KO groups. When measurements were restricted to two WT and two iNOS KO groups, comparisons were done among all groups. In some instances, the three WT groups were compared among themselves, and the same was done independently for the three iNOS KO groups, as indicated in the figures. For collagen content, comparisons were made only against the control WT by a two-tailed *t*-test. Differences were considered significant at $P < 0.05$.

Results

Glycemia was measured weekly in nonfasted animals under isoflurane anesthesia until it raised above a 350 mg/dL threshold in the STZ injected mice, and then immediately before sacrifice. At this point the control WT mice not injected with STZ showed a spontaneous high glycemia (225 ± 23 mg/dL), and to a lower extent this also occurred with the iNOS KO mice (188 ± 5 mg/dL) (Figure 1 top). This appears to be in part inherent to the C57Bl6J genetic background for both strains, where the WT develops a mild hyperglycemia with a regular diet and a marked hyperglycemia with a high-fat diet [43]. But in part it is artifactual too because of nonfasting and the transient effects of isofluorane anesthesia before sacrifice, as confirmed by the fact that when animals were not anesthesized with isofluorane and blood values were obtained after fasting, they were only moderately high in both strains (120–130 mg/dL). Daily insulin treatment normalized glycemia.

In order to corroborate that the nonfasting hyperglycemia in the non-STZ injected animals (control WT and iNOS KO mice) was artifactual, an oral glucose tolerance test in fasting animals was performed 1 week before sacrifice by administering a bolus of glucose and compared with injected the STZ-WT and STZ-iNOS KO animals (diabetic mice). The STZ-injected animals were treated daily with insulin except for the day of the assay, and the animals showed a

Figure 2 Genetic deletion of inducible nitric oxide synthase (iNOS) causes a reduction in the corporal smooth muscle (SM)/collagen ratio, intensified by diabetes and counteracted by insulin. Top panel: representative micrographies of tissue corporal sections for each group stained with Masson trichrome. Bottom panel: quantitative image analysis for the ratio between areas stained for SM (red) versus collagen (blue). WT = wild type mice; STZ = streptozotocin; WT + STZ = WT treated with STZ; WT + STZ + INS = WT + STZ treated with insulin; iNOS KO = iNOS knockout mice; iNOS KO + STZ = iNOS KO treated with STZ; iNOS KO + STZ + INS: iNOS KO + STZ treated with insulin. Values are means ± SEM. **$P < 0.01$; for WT + STZ vs. WT, and iNOS KO+STZ. #: $P < 0.05$ for iNOS KO vs. WT.
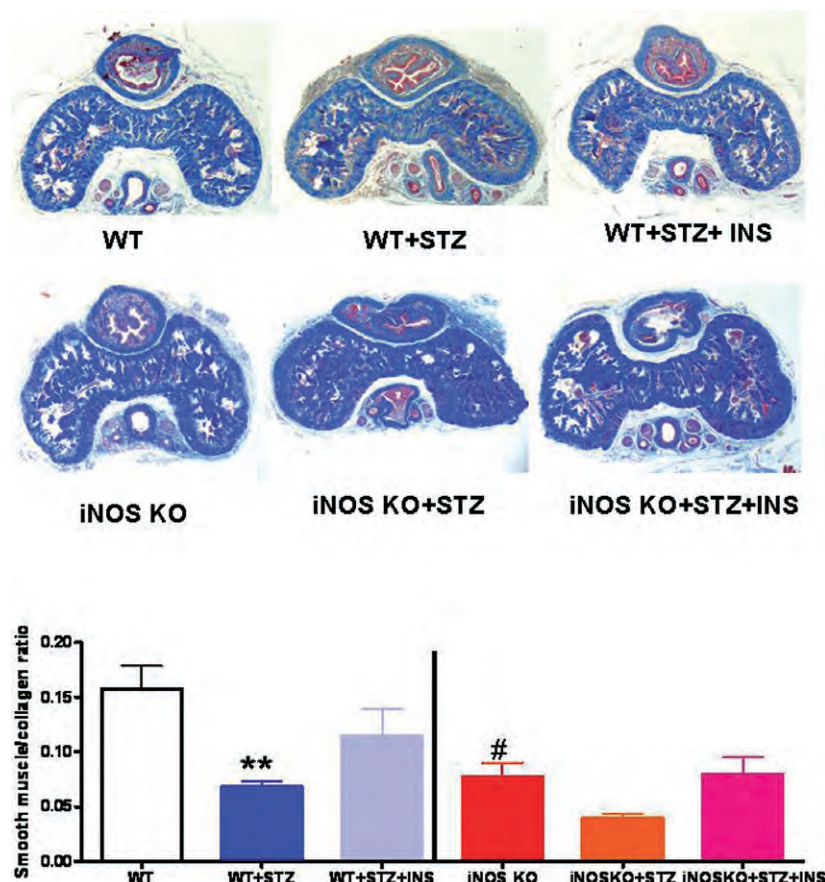
prolonged hyperglycemia lasting for at least 3 hours while the non-INS- or STZ-injected animals show a normal curve response with a decrease of the glycemic levels after 1 hour, and virtual normalization after 3 hours following the bolus administration (bottom). The latter animals will be referred to as "controls" and considered as nondiabetic.

The STZ single injection given to the WT mice virtually doubled the nonfasting glycemia values, making the mice frankly diabetic, and as expected insulin treatment normalized these levels. It took a high dose of STZ for the iNOS KO animals (also given to the controls) to become diabetic, in agreement with the relative resistance of this strain to STZ-induced hyperglycemia reported at lower doses [44]. Proteinuria increased up to eightfold in the STZ-treated animals as compared with controls, but this was completely counteracted by insulin (not shown). No ketonuria was detected in the diabetic animals, and the specific gravity and pH of the urine was similar among all groups. Body weights were reduced by STZ in the WT mice (24.0 ± 1.2 vs. 30.3 ± 0.7 in the noninjected

controls), and this reduction was partially prevented by insulin, but in the iNOS KO there were no significant changes in body weights by either STZ or STZ and insulin administration (not shown).

Analysis of the corpora cavernosa tissue sections by Masson trichrome staining revealed that there was a considerable reduction of the SM to collagen ratio in the control iNOS KO mice as compared with the control WT mice (Figure 2), as shown by the representative micrographies (top) and QIA (bottom). The hyperglycemia induced by STZ led to a nearly 50% decrease in the WT mice and to a similar but nonsignificant decrease in the iNOS KO mice. Insulin, which had normalized glycemia, also virtually prevented the hyperglycemia-induced reductions in the SM/collagen ratio.

These effects were confirmed by immunodetection of ASMA as a marker of corporal SM cells by both visual inspection (Figure 3 top) and QIA (bottom), except that there was no significant difference upon STZ treatment between the control iNOS KO and the control WT. The decrease in SM cell content in the WT-STZ-treated animals
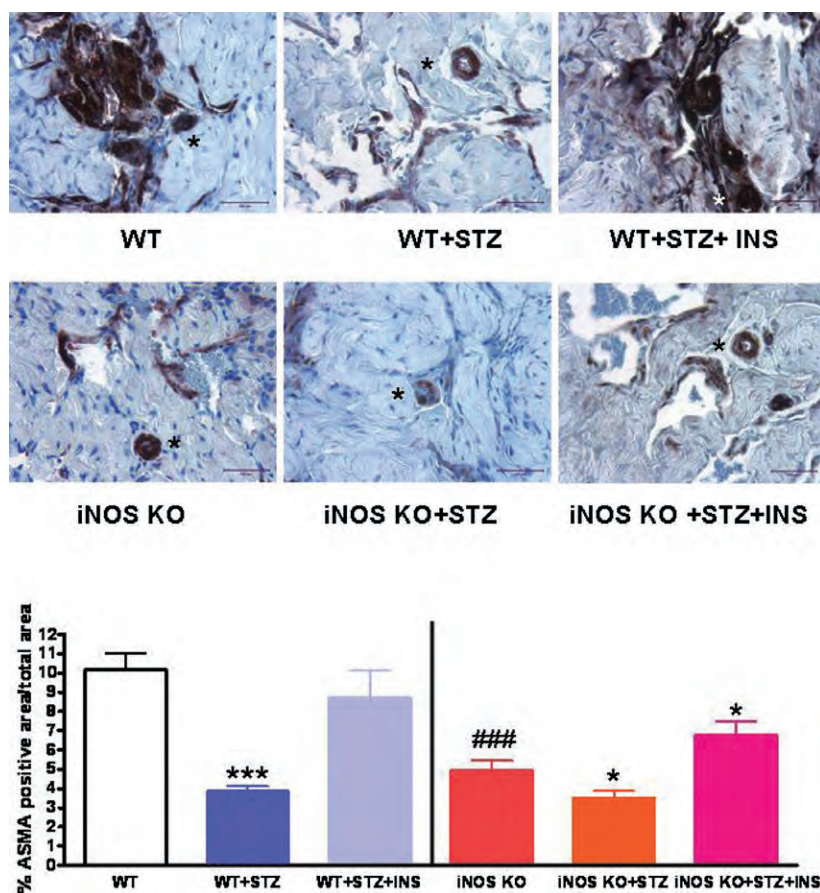
Figure 3 Genetic deletion of inducible nitric oxide synthase (iNOS) causes a reduction in the content of corporal smooth muscle (SM), intensified by diabetes and counteracted by insulin. Top panel: representative micrographies of tissue corporal sections for each group immunostained for α-smooth muscle actin (ASMA). Bottom panel: quantitative image analysis for the ratio between the area stained for SM (brown) vs. the total area. WT = wild type mice; STZ = streptozotocin; WT + STZ = WT treated with streptozotoci STZ; WT + STZ + INS = WT + STZ treated with insulin; iNOS KO = iNOS knockout mice; iNOS KO + STZ = iNOS KO treated with STZ; iNOS KO + STZ + INS = iNOS KO + STZ treated with insulin. Values are means ± SEM. ***$P < 0.001$; *$P < 0.05$ for WT + STZ vs. WT + STZ + INS and WT; and iNOS KO + STZ and iNOS KO + STZ + INS vs. iNOS KO. ###$P < 0.001$ for iNOS KO vs. WT.

vs. their respective controls was in perfect agreement with the apoptotic index determined by TUNEL (Figure 4). A similar situation occurred with the restoration of SM cell number and the decrease in the apoptotic indexes in the mice treated with insulin. However, the absence of iNOS counteracted the increase of apoptosis by diabetes seen in the WT mice.

The cell proliferation index determined indirectly by immunostaining for PCNA was doubled in the iNOS KO control as compared with the WT control, but STZ injection did not cause any significant change in any of them (Figure 5 top). As a result, the cell turnover measured by the ratio between proliferation and apoptosis was surprisingly higher in the control iNOS KO vs. the WT (4.0 vs. 1.1) and in the STZ-treated animals (2.8 and 6.4, respectively) vs. the corresponding controls.

As the previous results showed that insulin prevents the histopathological changes seen in the diabetic corpora, no further determinations were considered necessary for the insulin-treated animals in all the remaining assays. Hydroxyproline determinations in fresh corporal tissue for collagen content expressed in ug collagen/g fresh tissue showed a significant but modest (14%) increase in the control iNOS KO vs. the control WT (4.36 ± 0.29 vs. 3.06 ± 0.30; $P < 0.05$). STZ injection did not change the control value for the iNOS KO (4.37 ± 0.17), and the increase in the value for the WT was marginally nonsignificant (4.35 ± 0.50; $P = 0.06$).

The corporal expression of a key pro-fibrotic factor, TGFβ1, determined by immunohistochemistry, was not affected by the genetic ablation of iNOS expression, but it was considerably increased by STZ-induced diabetes (Figure 6). Similarly, oxidative stress as denoted by XOR expression in the corporal tissue was marginally higher in the control iNOS KO vs. the WT, but it was the STZ injection in the iNOS KO that induced a remarkable increase, virtually doubling the value (Figure 7). In contrast, as in the case of TGFβ1, diabetes in the WT mice was not associated with an increase in oxidative stress.

Figure 4 Genetic deletion of inducible nitric oxide synthase (iNOS) protects against the apoptosis caused by diabetes in the wild type (WT) mice that is counteracted by insulin. Top panel: representative micrographies of tissue corporal sections for each group immunostained for apoptotic bodies by the TUNEL reaction. Bottom panel: quantitative image analysis for the ratio between the number of apoptotic cells over the total cells counterstained with hematoxylin. STZ = streptozotocin; WT + STZ = WT treated with STZ; WT + STZ + INS = WT + STZ treated with insulin; iNOS KO = iNOS knockout mice; iNOS KO + STZ = iNOS KO treated with STZ; iNOS KO + STZ + INS = iNOS KO + STZ treated with insulin. Values are means ± SEM. *$P < 0.05$ for WT + STZ vs. all others.
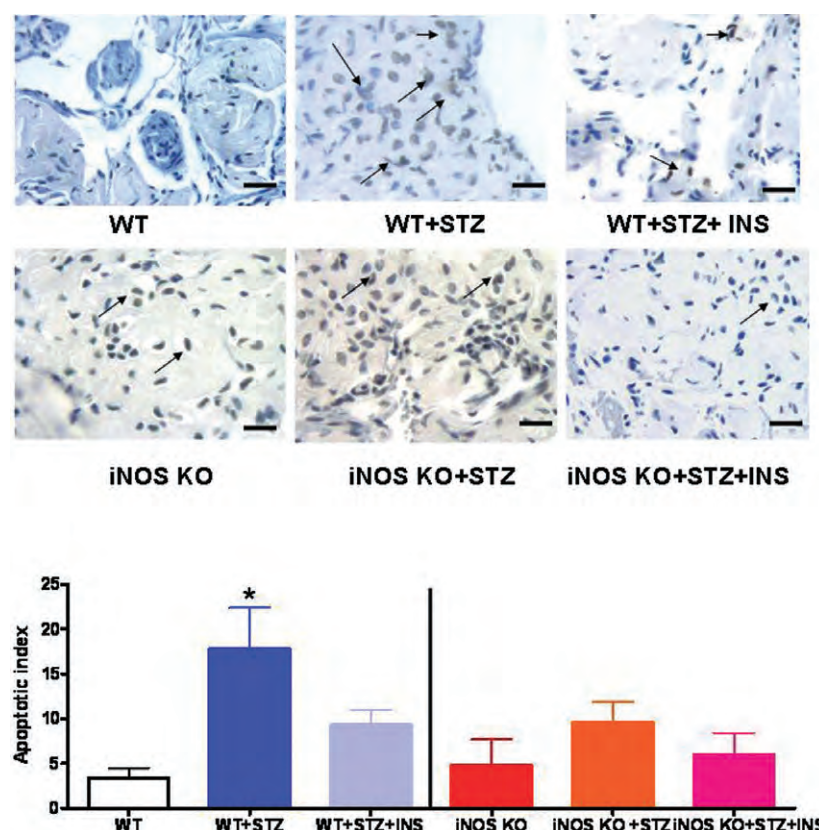
Discussion

This is the first report on the characterization of histopathological and biochemical changes in the corpora cavernosa induced singly by iNOS genetic inactivation or in combination with diabetes. The current results confirm our claims that (i) the loss of SM and the induction of fibrosis and oxidative stress in the corpora are major factors in causing its lack of compliance in diabetes, resulting in ED; and (ii) iNOS spontaneous induction is in this setting a defense mechanism against fibrosis rather than a deleterious factor because the lack of iNOS induced more fibrosis in both the control and diabetic animals. Therefore, in the specific case of the penis of the diabetic iNOS KO, the genetic inactivation of iNOS expression confirms previous results of iNOS inhibition or overexpression in WT rats [3,19,22,23] and provide a more coherent view on the postulated iNOS protective antifibrotic role than the confusing literature in other organs where iNOS is either beneficial in this respect [27–30,37,38] or inactive or noxious [31–36], according to experimental models and conditions. This is not surprising, knowing the biphasic and often antagonistic effects that nitric oxide exerts in many processes that depend on local tissue concentrations and cell types [45,46], and that are in a way replicated with one of the main pro-fibrotic factors, TGFβ1 [47,48].

We do not believe that the antifibrotic and SM protective effects of iNOS in the penile tissues are due to any fundamental difference with its response in cardiac and renal tissues. First, only the studies involving the genetic deletion or the pharmacological inhibition of iNOS may provide a meaningful answer in this respect, and many of those cited above indicate that iNOS is also protective in myocardial infarction or diabetic nephropathy. Second, leaving aside differences in experimental approaches, such as the experimental condition that elicits the fibrosis and the time frame when it is studied, the most important factors appear to be the intrinsic features of the fibrotic development in each organ and the nature of the key target cells.

The drastic and abrupt onset of fibrosis in myocardial infarction, or the progressive and relatively fast development of renal tubulointerstitial fibrosis in type 2 diabetes, differ from the much milder and slower process occurring with aging or diabetes in the penis. As a result, the intensity and duration of
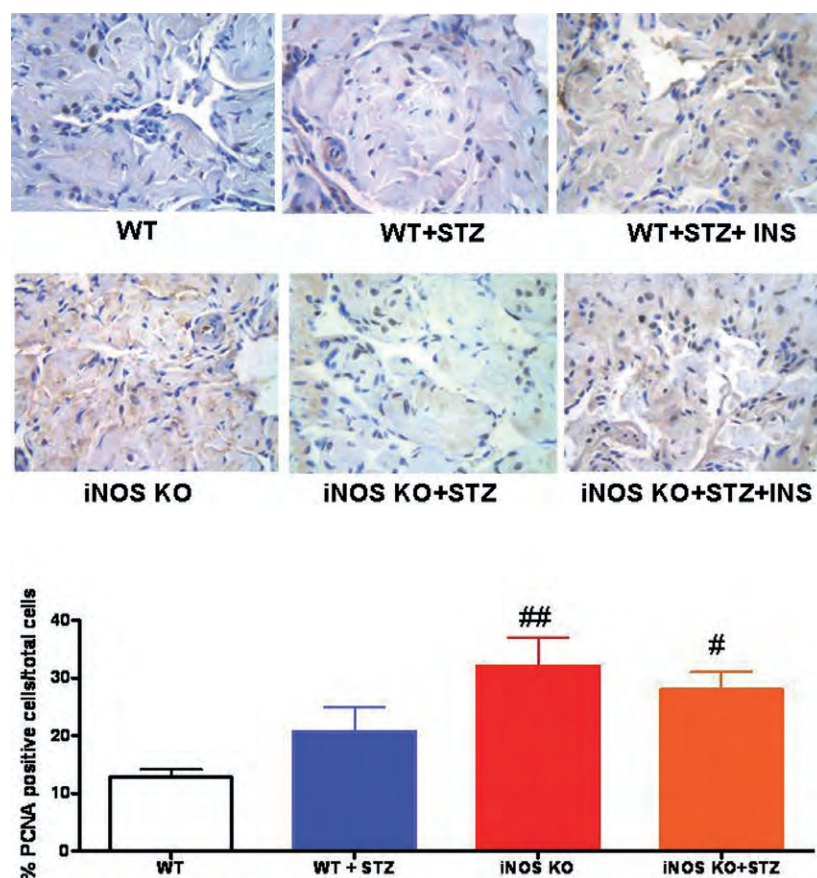
Figure 5 Genetic deletion of inducible nitric oxide synthase (iNOS) stimulates corporal cell replication, and diabetes does not affect it. Top panel: representative micrographies of tissue corporal sections for each group immunostained for proliferating cells by immunostaining for proliferating cell nuclear antigen (PCNA). Bottom panel: quantitative image analysis for the ratio between the number of proliferating cells over the total cells counterstained with hematoxylin. WT = wild type mice; STZ = streptozotocin; WT + STZ = WT treated with STZ; iNOS KO = iNOS knockout mice; iNOS KO + STZ; iNOS KO treated with STZ. Values are means ± SEM. ##$P < 0.01$; #$P < 0.05$ for iNOS KO vs. WT and iNOS KO + STZ vs. WT + STZ.

the inflammatory phase where iNOS is initially produced, and the release of profibrotic cytokines and reactive oxygen species that counteract the beneficial effects of iNOS, are also different, and this may allow in some cases the predominance of iNOS pro-apoptotic effects as previously reported in the brain [49].

The loss of SM in the corpora and the development of fibrosis and apoptosis in the WT mouse caused by hyperglycemia induced by STZ agree with previous results in rat, mouse, and rabbit models of type 1 diabetes [50–52]. It is logical to assume that these changes are the main cause of the impairment of corporal compliance seen in these models, both in organ bath and *in vivo* in the response to electrical field stimulation [53,54]. In contrast, the relative contribution of corporal endothelial dysfunction, extrapolated from the prevailing views in vascular atherosclerosis research, has not been histologically or mechanistically evaluated conclusively in impotent men or animal models of ED [55–57]. It is surprising that long-term insulin treatment of the WT mouse, which normalized glycemia, was only partially effective to prevent excessive collagen deposition

and apoptosis. This implies that hyperglycemia itself may not be the main inducer of fibrosis and oxidative stress in the corpora cavernosa in this model of type 1 diabetes, or that the intermittent control of glucose due to daily injections was not sufficient to produce a total recovery from the histopathological and biochemical changes induced by diabetes. Corporal damage may also result from a neuropathy of multifactorial origin, and the picture may be even more complicated in models of type 2 diabetes and morbid obesity [6–17].

The fact that iNOS deletion per se caused corporal SM loss and fibrosis in nondiabetic animals and intensified them when diabetes was induced by STZ (that also elevated apoptosis and TGFβ1 expression as compared with nondiabetic controls) agrees with our prior results regarding iNOS expression in other models [3,19,23,24] and with some studies in other organs [27–30]. It is noteworthy that oxidative stress measured by XOR levels in corporal tissue was not elevated by diabetes alone in the WT mouse but it was in the iNOS KO, thus reinforcing the assumption that iNOS in this setting may act as an antioxidant by sequester-

ing ROS through the reaction with nitric oxide to form peroxynitrite [21].Unfortunately, the expression and role of iNOS in the human corpora cavernosa, from the initial description of its induction in human penile SM cells and the cloning of its cDNA [22,58,59], have not been further studied other than for its immunohistochemical detection in some patients with diabetes [60]. However, it has been well characterized in the human Peyronie's disease plaque affecting the tunica albuginea, where its antifibrotic role was first established [24–26], even if this role was not initially acknowledged [61].

In summary, the general antifibrotic and antioxidative stress role of iNOS in the penile corpora
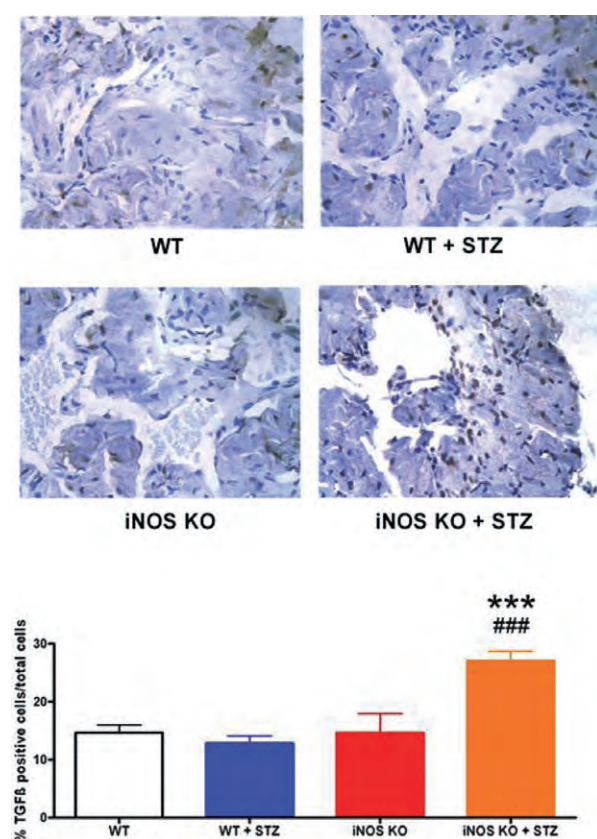
Figure 6 Genetic deletion of inducible nitric oxide synthase (iNOS) causes an increase in corporal TGFβ1 expression only in diabetes. Top panel: representative micrographies of tissue corporal sections for each group immunostained for TGFβ1 (insulin treatments not represented). Bottom panel: quantitative image analysis for the ratio between the number of cells positive for TGFβ1 and the total cells counterstained with hematoxylin. WT = wild type mice; STZ = streptozotocin; WT + STZ = WT treated with STZ; iNOS KO = iNOS knockout mice; iNOS KO + STZ = iNOS KO treated with STZ. Values are means ± SEM. ***$P < 0.001$ for iNOS KO + STZ vs. iNOS KO. ###$P < 0.001$ for iNOS KO + STZ vs. WT + STZ.
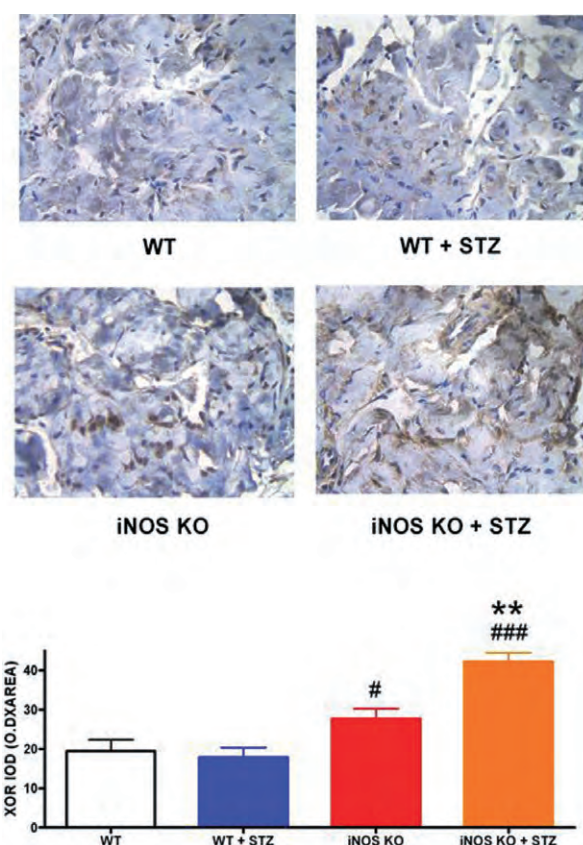
Figure 7 Genetic deletion of inducible nitric oxide synthase (iNOS) does not affect corporal oxidative stress, but diabetes does induce it. Top panel: representative micrographies of tissue corporal sections for each group immunostained for oxidative stress by immunostaining for xanthine oxidoreductase (XOR). Bottom panel: quantitative image analysis for the ratio between the positive vs. total areas expressed in integrated optical density (IOD). WT = wild type mice; STZ = streptozotocin; WT + STZ = WT treated with STZ; iNOS KO = iNOS knockout mice; iNOS KO + STZ; iNOS KO treated with STZ. Values are means ± SEM. **$P < 0.01$ for iNOS KO + STZ vs. iNOS KO. ###$P < 0.001$; #$P < 0.05$ for iNOS KO vs. WT and iNOS KO + STZ vs. WT + STZ.

cavernosa previously shown for aging and cavernosal nerve damage was confirmed and extended to type I diabetic animals by showing that the inactivation of the iNOS gene exacerbates corporal fibrosis in diabetes.

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Treatment of Peyronie's disease with PDE5 inhibitors: an antifibrotic strategy

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Abstract | Peyronie's disease (PD) is a localized fibrotic condition of the tunica albuginea that is associated with risk factors for corpora cavernosa fibrosis (such as advanced age and diabetes) and Dupuytren contracture, another localized fibrotic process. Most of the current pharmacological treatments for PD are not based on antifibrotic approaches that have shown promising results in animal models and clinical efficacy in other fibrotic conditions, which may explain why they are generally unsuccessful. Evidence gathered in human specimens and animal models of PD have elucidated aspects of its etiology and histopathology, showing that overexpression of transforming growth factor β1, plasminogen activator inhibitor 1, reactive oxygen species and other profibrotic factors, which are, in most cases, assumed to be induced by trauma to the tunica albuginea, leads to myofibroblast accumulation and excessive deposition of collagen. At the same time, a steady overexpression of inducible nitric oxide synthase, leading to increased nitric oxide and cGMP levels, seems to act as an endogenous antifibrotic mechanism. This process has also been reported in corporal and cardiovascular fibrosis, and has led to the demonstration that long-term continuous administration of phosphodiesterase type 5 inhibitors counteracts the development of a PD-like fibrotic plaque in a rat model, and later extended to the prevention of corporal fibrosis in animal models of erectile dysfunction.

Introduction

Peyronie's disease (PD) is somewhat of a misnomer, as it is actually restricted to a small localized fibrotic plaque of the tunica albuginea of the penis.[1,2] Epidemiologic studies suggest that the disease may be present in up to 10% of all men, but primarily affects those in their sixties and seventies.[1–6] The reason PD attracts attention is that many men with the disease have some form of erectile dysfunction, and in the erect state the afflicted organ tends to curve and may be painful during intercourse. Despite its typical fibrotic histopathology, this condition is not associated with other localized or diffuse fibrotic processes, with the single exception of Dupuytren contraction, with which it shares a similar histopathology.[7–9] No satisfactory medical treatments for PD are currently available; however, experimental models have provided new insights into its pathophysiology and etiology, which have facilitated the investigation of alternative therapeutic approaches, including long-term continuous administration of phosphodiesterase type 5 (PDE5) inhibitors[10] as an antifibrotic modality. In this Review we examine the experimental evidence that forms the basis for this treatment strategy. No reports of the clinical efficacy of long-term PDE5 inhibition in patients with PD have been published, and, although the first preliminary animal study dates back to 2003,[11] this should still be considered as a novel management approach that requires future clinical validation.

Competing interests
The authors declare no competing interests.

Pathophysiology

A widely accepted hypothesis on the etiology of the PD plaque is that it originates from trauma or microtrauma to the erect penis, primarily during different types of sexual activities.[12] This hypothesis is based mainly on the demonstration of fibrin staining or immunodetection in tissue sections of human PD plaques,[13–15] findings that have been corroborated in an animal model of PD.[14] A plausible interpretation of this hypothesis is that the fibrin originates from fibrinogen that has extravasated into the interstices of the tunica albuginea during a traumatic sexual episode. Inhibition of the fibrinolytic system or an inability to degrade the intravasated fibrin would then lead to its persistence in the tunica, which initially leads to an acute inflammatory response. Because the fibrin is not degraded, the protein continues to exert a proinflammatory response, which ultimately leads to an abnormal healing process. The end result is the formation of a 'scar' that at some time evolves into a palpable plaque.[16] Indeed, injection of fibrin directly into the tunica albuginea of the rat penis elicits a PD-like plaque, resembling in many aspects the histology and evolution of the human PD plaque.[14,15,17]

The epidemiological association of PD with a history of sexually elicited trauma of the penile or pelvic surgery, which may affect the homeostasis of tissues in the penis, supports the trauma-related hypothesis for at least part of the patient population,[8,18,19] despite some contradictory evidence.[5,8,20] Considering the association of PD with Dupuytren contracture, genetic or immune-related predisposition to PD may modulate the tunical healing

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Key points

- Rodent models of Peyronie's disease (PD) are representative of most of the main histological and biochemical features present in human specimens

- Cell cultures obtained from the human PD plaque and its rat counterpart have added to the experimental evidence acquired in the human and in animal models

- Endogenous mechanisms of defense against tunical tissue inflammation, oxidative stress and fibrosis have been detected in the PD plaque and the rat PD-like lesion, and may be mimicked pharmacologically for treatment

- Endogenously elicited inducible nitric oxide synthase leads to sustained production of nitric oxide and cGMP, which counteract myofibroblast differentiation, accumulation of reactive oxygen species, cytokine release, and collagen deposition

- Continuous long-term administration of nitric oxide donors and phosphodiesterase 5 inhibitors has shown preventive and corrective effects in a rat model of PD: studies in patients with PD are now needed

reaction following any type of trauma to the penis, but this possibility has not been studied as intensively as is needed.[21–23]

The pathology of the PD plaque has been investigated in a variety of studies in human and animal model specimens and in related cell cultures.[1,10] Based on the results of histochemistry, immunohistochemistry and other assays performed on the human PD plaque tissues, it is clear that fibrosis—the excessive deposition of collagen and extracellular matrix (ECM) with disorganization of collagen fibers and loss of elastic fibers—is the main pathological process, combined in most cases with fibrin accumulation and different degrees of inflammation.[12–14,24,25]

Myofibroblasts (cells that share the fibroblast and smooth muscle phenotypes[26,27]) are not normally present in the tunica albuginea of the penis, but have been identified as the cells responsible for the disarrangement of the ECM in the PD plaque.[28–32] The normal process of apoptosis that eliminates myofibroblasts after they have fulfilled their role in wound healing is somehow inhibited in PD, thus leading to their persistence in the tunica albuginea. This myofibroblast accumulation is common not just to scar formation in the skin or the infarcted heart, but to most other types of fibrosis.[26,27] The PD plaque becomes harder by progressing through an intensification of fibrosis (with or without the persistence of inflammation) and, in at least 15% of the patients, through an advanced stage of calcification and ossification involving osteoblasts.[33,34] Spontaneous regression of the plaque after its initial formation occurs in rare cases.[9,34]

The scattered evidence regarding human PD plaque tissues has been considerably expanded by systematic approaches in experimental animal models, mainly in the widely used rat model of PD induced by transforming growth factor β1 (TGF-β1).[1,10] This key profibrotic factor, present in multiple tissues[35] and produced in the human PD plaque, is found at increased levels in the blood of PD patients.[36] Similarly, when a peptide derived from the TGF-β1 sequence is injected into the rat tunica, a plaque resembling that seen in human PD is found around 45 days later[11,37–41] at the injection site. Other less frequently employed but nonetheless useful animal models are based on either the successive injections of an adenoviral construct expressing a constitutively active TGF-β1 protein, leading to penile curvature during the erect state and, at times, calcification within the plaque,[42] or on a single fibrin injection that mimics the extravasation of fibrinogen, initiating acute inflammation followed by the rapid development of the PD-like plaque.[14,15,17] Both TGF-β1 and myostatin, another profibrotic factor within the TGF-β family, are involved in this process,[41] and it is quite likely that the key downstream signaling occurs via the Smad pathway, which is the mechanism common to most factors in this family.[43] A tight skin (Tsk) mouse model has been described that develops a spontaneous PD-like plaque with penile bending and areas of chondroid metaplasia with heterotypic ossification.[44]

These animal models, therefore, represent most of the histologic and biochemical features of the human PD plaque, including inflammation, myofibroblast accumulation, collagen deposition, oxidative stress, calcification, ossification and penile bending, among others. Thus, we believe that the PD-like lesion, either elicited experimentally or by spontaneous mutations in the rodent tunica, is more complex than a mere tunical fibrosis event, and, imperfect as most disease models in laboratory animals are, this experimental plaque is adequate for preclinical testing of various therapeutic strategies for PD.

Finally, the use of cell cultures from the normal, myofibroblast-free tunica albuginea or from the human PD plaque or the induced PD-like plaque from a rat, which are enriched in myofibroblasts, has allowed us to more precisely define the role of myofibroblasts in the pathophysiology of PD.[28–32,45,46] These cells have been shown to be responsible for the excessive collagen deposition seen in PD, and have even been postulated to cause penile bending by their contractile features. Moreover, pluripotent stem cells have been identified in the PD cultures. This may explain the fibrotic and osteogenic progression of the PD plaque upon the release of cytokines following microtrauma to the penis, which would stimulate stem cell commitment to this cell lineage.[31,45] PD fibroblasts are also potentially tumorigenic, or acquire this trait upon culture, but it is not known whether this is related to the presence of stem cells.[47]

There is no doubt that cell cultures derived from the human PD plaque and normal tunica albuginea closely represent their respective histologic features, notwithstanding the obvious shortcomings of any type of cell culture compared to the *in vivo* tissue. This has been tested with a multiplicity of immunocytochemical and western blot markers, as well as DNA microarrays and reverse transcription polymerase chain reaction procedures for the detection of fibroblasts, myofibroblasts and stem cells, and their respective differentiation and roles in inflammatory and fibrotic processes. The situation is similar regarding cell cultures obtained from the rat PD-like plaque, which have been shown to mimic their human counterparts. All these cultures have been useful

tools for defining therapeutic targets at the cellular and molecular level.

Cellular and molecular mechanisms

Results from experimental studies that have employed a variety of cellular and molecular biology techniques in the PD models described above, combined with the information obtained from the analysis of the human PD plaque, have made it possible to define an overall mechanistic picture of the initiation and progression of the PD plaque.[48] The mechanism resembles that seen in some other localized fibroses, including the more gradual and diffuse type that occurs in the penile corpora cavernosa of men with erectile dysfunction and many animal models of this disorder.[49] The main features of PD fibrosis are described below.

Fibrinogen extravasated into the tunica albuginea of the penis accumulates at the site of the future PD plaque owing to inhibition of the fibrinolytic and other proteolytic systems, primarily due to overexpression of plasminogen activator inhibitor 1 (PAI-1). The resulting fibrin formation, and possibly with the assistance of immunoglobulins and other extravasated proteins, triggers the release and/or activation of TGF-β1, PAI-1, and reactive oxygen species (ROS), which are recognized as key profibrotic factors in many tissues, including the kidney and vascular system. Concurrent expression of other cytokines, including monocyte chemotactic protein 1 (MCP-1; also known as CC-chemokine ligand 2 [CCL-2]), which is associated with acute inflammation that often progresses to a chronic phase, overexpression of other members of the TGF-β1 family (such as myostatin) and components of their common Smad signaling pathway, and other unknown agents combine to elicit the fibrotic process. The PD plaque then develops through excessive collagen deposition, elastin degradation, myofibroblast differentiation from fibroblasts or stem cells in the tunica, oxidative stress, and eventually calcification (Figure 1).[10,48,50,51]

The accumulation of tissue inhibitors of metalloproteinases (TIMPs) and the relative inhibition of collagenases (and/or a possible downregulation of their expression), which interferes with the normal breakdown of the accumulated collagen—and potentially, in the case of TIMPs, with therapeutic collagenase delivered to the plaque—contribute to the maintenance of the fibrotic process.[52]

One of the main findings stemming from DNA microarray analysis of the molecular profile of the PD plaque is the recognition that this tissue may be undergoing constant cellular and molecular turnover, and that spontaneous development of defense mechanisms to counteract fibrosis and oxidative stress might occur.[53,54] This transcriptional analysis detected overproduction of matrix metalloproteinases (MMPs) 2 and 9 (which contribute to collagen breakdown), decorin (which binds and neutralizes TGF-β1), and thymosins (which activate MMPs) in both PD plaques and Dupuytren nodules, as well as in cell cultures of these tissues. All these proteins seem to act as antifibrotic agents, either by combating
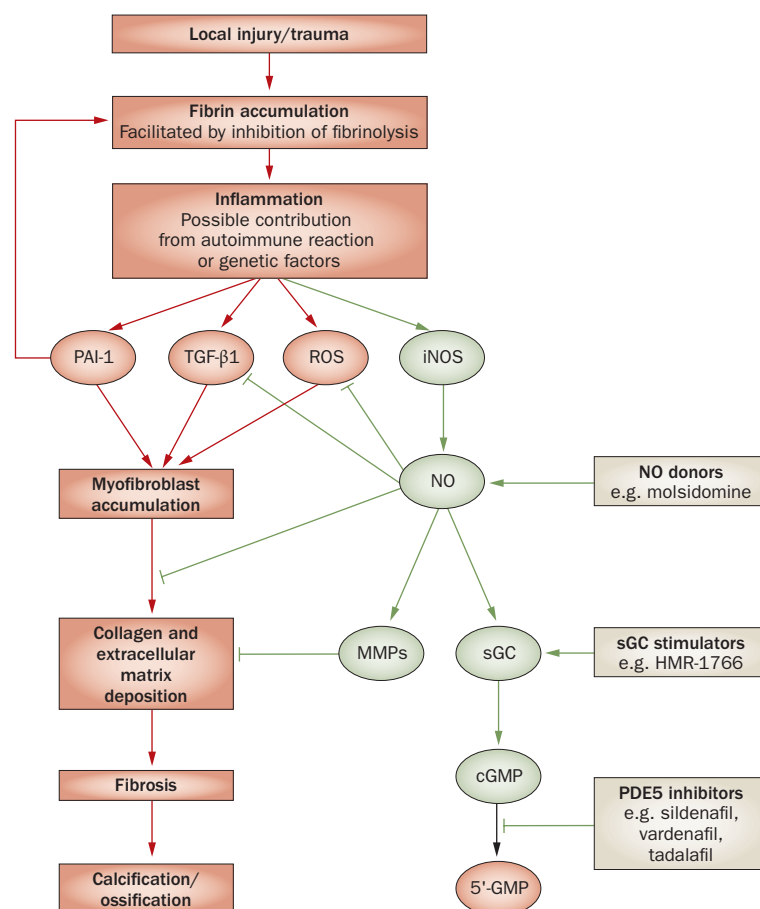
Figure 1 | Schematic representation of the etiology and pathophysiology of the fibrotic process leading to the Peyronie disease plaque (red), and the antifibrotic effects (green) of endogenous iNOS. Fibrin extravasated after injury or trauma to the penis accumulates in the tunica albuginea, which induces a local proinflammatory response. This leads to increased production of several key profibrotic factors, including PAI-1 (which inhibits fibrinolysis), TGF-β1 and ROS, which contribute to the accumulation of myofibroblasts. Myofibroblasts are responsible for the increased collagen and extracellular matrix deposition leading to fibrosis, and, owing to inhibited apoptosis of these cells, persist in the tunica albuginea. The production of iNOS seems to be an endogenous antifibrotic factor, leading to increased levels of NO in the affected tissue. NO inhibits collagen synthesis and myofibroblast differentiation and reduces oxidative stress by quenching ROS. In addition, NO increases the activity of sGC, which in turn increases levels of cGMP, which has further antifibrotic effects. Potential pharmacological interventions (gold) that might prevent or partially reverse plaque formation include NO donors, sGC stimulators and PDE5 inhibitors. Abbreviations: iNOS, inducible nitric oxide synthase; MMPs, matrix metalloproteinases; NO, nitric oxide; PAI-1, plasminogen activator inhibitor 1; PDE5, phosphodiesterase type 5; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; TGF-β1, transforming growth factor β1.

collagen deposition or promoting its breakdown; therefore, it is plausible to postulate that they are produced in response to the fibrotic processes and that progression depends on the balance between the noxious and protective mechanisms, which in some cases may lead to spontaneous regression of the plaque.

Role of inducible nitric oxide synthase

Despite the experimental evidence outlined above, the current pharmacological management of PD is mostly

empirical, as it is generally based on the use of drugs targeting nonspecific or ancillary aspects of PD, such as inflammation or cell replication.[2,55] Virtually nothing has been translated from the abundant pharmacological studies in other types of fibrosis, or from preclinical studies in animal or cell culture models of PD. This renders PD a sort of orphan disease in terms of a scientifically rational approach to therapy, in contrast to the other types of fibrosis where, in general, clinical use is supported by promising preclinical studies.[48,56]

However, among the putative endogenous mechanisms of defense against fibrosis that are postulated to operate in the PD plaque, the most intensively studied and highly promising in terms of therapeutic potential is the spontaneous induction of inducible nitric oxide synthase (iNOS), a NOS isoform that is not expressed in normal penile tissue.[57] Whereas in the past it was assumed that the presence of iNOS portended a deleterious outcome to a tissue, it is now believed to in fact be a protective mechanism against tissue fibrosis in certain settings. iNOS expression produces a steady output of nitric oxide, a compound that directly inhibits collagen synthesis and myofibroblast differentiation, quenches ROS via the production of peroxynitrite, and inhibits the TGF-β/Smad signaling pathway, thus counteracting fibrosis.[11,17,25,30,41] The pro-apoptotic effects of nitric oxide might also contribute to reducing the myofibroblast population. Remarkably, iNOS production in the penis is not restricted to the tunica albuginea or to PD. For example, iNOS is detectable in the corpora cavernosa of patients with diabetes, advanced age, and even following radical prostatectomy, where fibrosis of the corpora leads to the development of corporal veno-occlusive dysfunction (CVOD). iNOS production may also been seen in the penile arteries in disease states where arteriosclerosis or arterial stiffness is present.[57–64] In all these scenarios, iNOS production, resulting in some cases from an inflammatory process, is presumed to be an antifibrotic response to the development of fibrosis within these individual tissues.

Collectively, several lines of evidence in rodent models support the antifibrotic role of iNOS in the PD plaque and in corporal and vascular tissue. Gene transfer of iNOS cDNA, which then becomes constitutively expressed, reduces fibrosis in the tunica albuginea and corpora of the penis, whereas long-term, specific inhibition of iNOS activity (by the iNOS inhibitor L-NIL) counteracts this process in both tissues, as well as in the arterial wall.[17,25,58,60] Furthermore, genetic inactivation of the iNOS gene in the iNOS knockout mouse intensifies collagen deposition in the corpora in a process exacerbated by diabetes.[64] This agrees with what has been shown in other tissues in the iNOS knockout mouse model, in which the absence of iNOS increases interstitial fibrosis after unilateral ureteral obstruction and hepatic fibrosis in animals fed a high-fat diet and in those with streptozotocin-induced diabetic nephropathy.[65–70] iNOS also has a cardioprotective role in preconditioning during ischemia reperfusion injury in mouse kidney and in granulomatous disease.[71,72]

Some evidence suggests that iNOS deletion seems to be protective rather than detrimental in certain types of fibrosis,[73–76] and that iNOS overexpression is associated with increased fibrosis, particularly in the diabetic kidney.[77] However, nitric oxide is known to inhibit myofibroblast differentiation via inhibition of the TGF-β/Smad pathway, and to have general antifibrotic effects via inhibition of collagen synthesis and ROS quenching.[78–82] The protective effects of iNOS depend, therefore, on the specific tissue type and the pathological conditions under which it is induced. In the case of the penile tissues, including the PD plaque, all the evidence so far obtained supports an antifibrotic role for iNOS.

Treatment with PDE5 inhibitors

Although some of the beneficial, antifibrotic effects of iNOS are directly attributable to nitric oxide, others may result from the increased levels of cGMP produced following stimulation of guanylate cyclase by nitric oxide, which subsequently leads to protein kinase G activation. cGMP, and in some cases PDE5 inhibitors, have been shown to inhibit myofibroblast formation in cell cultures of human and rat PD plaques[30,32] and in lung fibroblasts.[83] These antifibrotic effects are also exercised by guanylate cyclase stimulators via protein kinase G stimulation and inhibition of fibrotic mediators such as angiotensin II, or by TGF-β or Rho activation.[83–87]

An early preliminary study in the rat model of TGF-β1-induced PD demonstrated that both oral sildenafil, a PDE5 inhibitor that protects cGMP from breakdown, and oral pentoxifylline, a predominantly PDE4 inhibitor that increases cAMP synthesis, counteract the development of the PD-like plaque.[11] In the case of pentoxifylline, it was proposed that the well-known cAMP–cGMP signaling crosstalk may be responsible for its antifibrotic effects, although direct effects of cAMP or the involvement of alternative pathways modulated by pentoxifylline can not be excluded. This study revealed a completely new mechanism of action for PDE5 inhibitors, in contrast to their standard on-demand clinical administration to facilitate penile erection upon sexual stimulation, which is mediated by their short-term relaxant effect on the corporal and arterial smooth muscle produced by a transient elevation of cGMP levels. The novel concept is that PDE5 inhibitors given for a sufficiently long time can induce a sustained elevation of nitric oxide and cGMP levels that, independently of their vasorelaxant effects, which would show only during sexual stimulation, act as antifibrotic agents by reducing collagen deposition, profibrotic factor release, oxidative stress and myofibroblast numbers.

In a subsequent study in the same rat model, it was shown that another PDE5 inhibitor, vardenafil, given orally and in different dosing regimens, not only prevented but partially reversed the formation of the PD-like plaque.[40] To test the early preventive effects of vardenafil, the drug was administered to male rats either in their drinking water or as a once-daily oral instillation at either 1 or 3 mg/kg per day for 45 days following a single injection of TGF-β1 into the tunica albuginea

to induce the PD-like plaque. Other animals, in which a PD-like plaque had already been formed, received either dose of vardenafil in their drinking water for 42 days (late, therapeutic administration). Preventive treatment at the higher dose (both continuous and once-daily treatments) reduced the overall collagen content, collagen III/I ratio and the number of myofibroblasts and TGF-β1-positive cells, and selectively increased the apoptotic index of cells (presumably including myofibroblasts), in the PD-like plaque. The lower dose was less effective. When vardenafil was given continuously in the drinking water for 42 days after the PD-like plaque was formed, a partial reduction in plaque size was observed. From these two studies,[11,40] it was concluded that long-term oral treatment with a PDE5 inhibitor slows and reverses the early stages of an experimental PD-like plaque in the rat, and might ameliorate a more advanced plaque.

The optimal therapeutic regimen for discontinuous oral administration of PDE5 inhibitors was not assessed in these studies, so whether oral instillation, perhaps at a higher dose, can regress an already formed plaque is not known. However, the authors discussed the possibility of testing combinations of PDE5 inhibitors and other compounds used for the treatment of PD, such as verapamil (a calcium channel blocker), vitamin E (an antioxidant) and collagenase. An important point that was made was that, owing to the multifactorial nature of fibrosis and the difficulty of reversing established collagen crosslinking, combination therapy might be more effective than a single agent when a well-formed PD plaque is present.[40]

This first demonstration of the antifibrotic effects of long-term, continuous administration of a PDE5 inhibitor was later extended to the corpora cavernosal fibrosis that underlies CVOD, caused either by aging or by neuropraxia secondary to cavernosal nerve resection, mimicking the post-radical-prostatectomy state.[59–62] In these cases, the effects of the three PDE5 inhibitors (sildenafil, vardenafil and tadalafil) on collagen deposition in the rat corpora were similar to those seen in the PD-like plaque; however, they also seemed to provide protection against the loss of smooth muscle cells, which are responsible for normal corporal compliance and their ability to relax and achieve normal veno-occlusion. In fact, the PDE5 inhibitors decreased corporal apoptosis—specifically of smooth muscle cells in this case, as opposed to the increased apoptotic index in tunical myofibroblasts observed in the PD plaque—and oxidative stress, thus preventing or correcting CVOD. Sildenafil prevented the progression of corporal fibrosis in penile histopathology induced by cavernous neurotomy in the rat and in patients who had undergone radical prostatectomy.[88–90] These antifibrotic effects of PDE5 inhibitors, specifically the prevention of collagen deposition and the inhibition of TGF-β1 expression and oxidative stress, were also seen in rat models of diabetic nephropathy, experimental glomerulonephritis, myocardial infarction and hypertrophy, and pulmonary fibrosis;[91–95] therefore, their antifibrotic effects do not seem to be restricted to

penile tissues. These effects should not be confused with the beneficial vasodilator mechanism exploited for the treatment of pulmonary hypertension.[96]

Despite the two experimental papers on the effects of continuous long-term treatment with sildenafil and vardenafil on the PD-like plaque in the TGF-β1 rat model,[11,40] the emerging literature on this modality in other types of tissue fibrosis, and the well characterized antifibrotic effects of cGMP and guanylate cyclase stimulators, no similar experimental studies have been performed in human patients with PD. An article related to the use of PDE5 inhibitors in patients with PD in fact focused on their standard "on-demand" application for treating erectile dysfunction, and not PD itself.[97] This lack of studies in humans does not seem to be due to concerns about potential adverse effects, as several trials have shown that daily administration of sildenafil or tadalafil is well tolerated.[98,99] Moreover, a 2006 case report described the beneficial effects of an antifibrotic regimen of drugs that upregulate nitric oxide (and, therefore, cGMP production) in two patients with refractory priapism (>48 h duration).[43] Based on the previous work in a rat model of PD,[11] the regimen included the PDE inhibitors pentoxifylline and sildenafil and the nitric oxide precursor L-arginine. At 1 year, both patients were found to have flexible corpora and no evidence of fibrosis.

Conclusions

Despite the strong preclinical evidence in animal models supporting the antifibrotic effects of continuous, long-term administration of PDE5 inhibitors in penile tissue, this approach has yet to be studied in patients with PD. The likelihood is that our wider experience of the on-demand use of PDE5 inhibitors for erectile dysfunction will eventually lead to the first clinical test of the antifibrotic hypothesis in the context of the relatively mild corporal fibrosis seen in patients after radical prostatectomy; only if successful in this application might its use be extended to PD. In any case, despite the promise of this novel approach, the progression of the human PD plaque to advanced fibrosis and calcification may restrict its application to the early stages of the disease. In addition, a combination regimen comprising PDE5 inhibitors and other agents that stimulate collagen breakdown may be needed to effectively reduce the size of an established plaque. We believe that a study in which the outcomes of men receiving a currently used treatment for PD plus a PDE5 inhibitor are compared with men receiving the same treatment plus placebo will help define the future role of PDE5 inhibitors in patients with PD.

Review criteria

We searched for original articles focusing on Peyronie's disease in PubMed published from 1980 onwards. The search terms we used were "Peyronie's disease" and "La Peyronie". All papers identified were full-text papers (unless indicated in the reference list) and were published in English, French or Spanish.

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Laboratory Forum

Experimental Models of Peyronie's Disease. Implications for New Therapies

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A B S T R A C T

Introduction. Despite its high prevalence and impact on the quality of life of patients, and that it is an excellent model for the study of fibrotic processes, Peyronie's disease (PD) is an orphan disease in biomedical research. The development of animal and cell culture models has advanced substantially the understanding of its molecular and cellular pathology and the proposal of new therapies.
Aim. To review the literature pertaining to the use of these models for the study of PD.
Methods. PubMed search conducted from the first report of an animal model for PD.
Results. This model, based on the finding that transforming growth factor β1 (TGFβ1) is overexpressed in the PD plaque, consists on the injection of TGFβ1 into the tunica albuginea of the rat. This leads to a PD-like plaque retaining many of the histological and biochemical features of human PD. Another rat model, based on the hypothesis that the PD plaque arises from trauma to the penis, causing fibrinogen extravasation that initiates as fibrin a fibrotic response, consists on injection of fibrin into the tunica. The cell culture model is based on the demonstration that myofibroblasts are abundant in the human PD plaque.
Conclusions. These models have: (i) clarified the role of microtrauma, myofibroblasts, and oxidative stress in plaque development; (ii) demonstrated that this tissue is under sustained turnover by fibrotic and antifibrotic mechanisms; (iii) showed the interplay of collagenolytic and fibrinolytic systems and their inhibitors; (iv) detected an endogenous antifibrotic process consisting of the expression of inducible nitric oxide synthase that counteracts oxidative stress, collagen synthesis, and myofibroblast generation; (v) characterized the antifibrotic effects of chronic treatment with phosphodiesterase type 5 (PDE5) inhibitors; (vi) discovered the cytogenetic instability of PD cells and alterations in their gene expression; and (vii) detected stem cells in the tunica albuginea with a potential role in fibrosis and ossification. **Gonzalez-Cadavid NF, and Rajfer J. Experimental models of peyronie's disease. Implications for new therapies. J Sex Med 2009;6:303–313.**

Key Words. Fibrosis; TGFβ; Penis; Myofibroblast; PDE5; Myostatin

Introduction

Except to urologists, Peyronie's disease (PD) [1–3] is a relatively unknown condition, although recent studies suggest that it may occur in up to 9% of the male population [4,5]. Because of its impact on male sexual health, PD can seriously affect the quality of life of patients and their partners [6]. Although it was described nearly 250 years ago, there still is no satisfactory medical treatment for PD. This is primarily because comparatively few research efforts have been focused

on the cellular and molecular pathology of this disorder, which is the prerequisite for finding potential therapeutic targets.

The fibrotic lesion or plaque of PD that develops in the tunica albuginea of the penis, causing penile curvature and very often pain and erectile dysfunction, recapitulates the sequence of events that characterize the development of tissue fibrosis in general. These are essentially an initial tissue insult (trauma, microtrauma, or local toxicity), followed by acute and then chronic inflammation that leads to deposition of excessive collagen and other extracellular matrix, fragmentation of elastin, and persistence of myofibroblasts. Fibrosis may then progress to partial ectopic calcification or ossification, a condition that is also seen in about 15% of PD patients [3]. In fact, before the advent of animal models for PD, and based purely on observations in human penile tissue, it was proposed that the PD plaque results from some form of trauma or microtrauma to the tunica albuginea, which allows for the accumulation of fibrin into the interstices of the tunica albuginea, ultimately leading to an abnormal wound healing process and formation of a scar [7,8]. Another seminal experimental finding was the report of increased levels of the profibrotic factor, transforming growth factor β1 (*TGFβ1*), measured within the PD plaque [9], an observation that led to the first experimental animal model for this disease.

The TGFB1 model was first proposed in 1997 by the University of California, San Francisco (UCSF) group led by Tom Lue, where they injected a TGFβ1-like peptide into the rat penile tunica albuginea [10] to produce a PD-like lesion. The second animal model for PD was described in 2003, when Davila et al. from University of California, Los Angeles (UCLA) reported the induction of a PD-like plaque similar to that of the UCSF group just by injecting fibrin itself into the tunica albuginea of the rat [11]. In addition, cell cultures from both the human PD plaque and normal tunica albuginea of the penis, originally described in 1982 by Somers et al. [12], were revisited and further characterized in 2000 by Mulhall et al. and have been proposed as an in vitro model to test some features of PD cells [13]. Taken together, these and subsequent studies on the animal and cell models performed within the last decade have helped to provide some key findings and insights into the etiology and molecular and cellular pathology of PD, and have led to the study

of some potential medical treatments based on novel mechanisms as discussed below.

The clinical significance of PD and the substantial advancements in the understanding of this disease, including the proposed novel therapies facilitated by these in vitro and in vivo models, have not as yet spurred substantial funding for basic translational research on these topics. However, it is hoped that this will soon change, primarily because it would be difficult to ignore the fact that PD turns out to be an excellent model to study the processes involved in tissue fibrosis. Considering how many human disorders ultimately involve some aspect of fibrosis, it is only a matter of time before scientists in other fields discover the importance of these models, not just for PD, but for fibrosis in general.

In the current article, we have reviewed studies that focused on the development and application of these experimental animal and cell culture models that were selected in PubMed under the key words "Peyronie's," "Peyronie," and even "La Peyronie," and were published over the last decade.

Experimental Models

Development and Early Characterization of the TGFβI Rat Model

Based on the fact that TGFβ1 is one of the main profibrotic factors in multiple tissues [14] and the observation of an increase in TGFβ1 expression in the PD plaque [9], Lue et al. injected various concentrations of a synthetic heptopeptide, named "cytomodulin," claimed as having TGFβ1 activity, into the tunica albuginea of rats. Animals were then sacrificed at 3 days, 2 weeks, and 6 weeks [10] following the cytomodulin injection. Histochemistry using Hart and trichrome stains revealed, in comparison with saline-injected tissues, that at 6 weeks, there was substantial chronic cellular infiltration, focal and diffuse elastosis, thickening of the tunica albuginea, disorganization and clumping of collagen bundles, and expression of TGFβ1, but not of the β2 and β3 isoforms, as determined by western blot. All concentrations of cytomodulin induced TGFβ1 mRNA expression after 2 weeks. These histological features seen in the rat tunica albuginea following the cytomodulin injection are characteristic of what is seen in the human PD plaque.

The problem for other investigators to duplicate or expand on these findings involving cyto-

modulin revolved around the fact that its amino acid sequence was not published [15]. However, a subsequent article from this same group showed that the recombinant full-length TGFβ1 protein produced similar effects on the tunica as the cytomodulin. In addition, the PD-like plaque induced by the TGFβ1 protein was reduced by colchicine, a drug claimed to be able to induce collagenase and to reduce myofibroblasts. This drug has been employed clinically for the early phase of PD [16]. The efficacy of a single injection of TGFβ1 to induce a chronic inflammation and fibrosis in the tunica is probably due to the fact that this factor is able to promote its own synthesis by feedback transcriptional stimulation. Very interestingly, surgical injury of the tunica albuginea, which should mimic microtrauma in men, could only induce histological changes similar to the acute phase of PD, but not to its chronic development, and the early up-regulation of TGFβ1 protein expression was transient [17].

The TGFβ1-induced model of PD was utilized by two other groups to clarify important aspects of the etiology and pathology of the fibrotic PD plaque. First of all, in 2000, the Tulane University group showed that at 6 weeks following TGFβ1 injection into the tunica albuginea of the rat, inducible nitric oxide synthase (*iNOS*) was induced to high levels of expression while constitutive NOS was decreased, as measured by western blots in the penile tissue homogenates [18]. In addition, penile erection induced either by electrical field stimulation of the cavernosal nerve (*EFS*) or by acetylcholine was reduced following formation of a PD-like plaque in the tunica. Since aminoguanidine, an iNOS inhibitor, enhanced the erectile response to EFS, it was concluded that iNOS induction occurring in this experimental setting was deleterious to corporal smooth muscle relaxation. However, it should be reminded that nitric oxide from iNOS, if acting at all on the erectile response, may possibly stimulate it [19], and that aminoguanidine can also inhibit other biological processes, such as the formation of advanced glycosylation end products [20,21], which contribute to fibrosis by creating collagen crosslinks. These caveats complicate the interpretation of the effects of iNOS expression on corporal tissue, and on the other hand do not clarify its role in the development of fibrosis in the tunica albuginea.

Subsequently, the same group supported their early study by showing that eNOS protein and gene expression were downregulated in the corpora cavernosa of the rats that developed a TGFβ1-induced plaque in the tunica albuginea, and that nitrotyrosine, an indicator of peroxynitrite formation, was considerably increased [22]. Peroxynitrite results from the reaction of nitric oxide with reactive oxygen species (*ROS*) formed during oxidative stress, and is pro-apoptotic, so that the inference continued to be that iNOS was deleterious to the corpora cavernosa. In fact, the authors showed that arginase II, the enzyme that cleaves L-arginine and therefore reduces the NOS substrate necessary to make nitric oxide, was considerably increased by the TGFβ1 injection into the tunica albuginea, and claimed that arginase II was also induced by iNOS.

Characterization of the Role of the Nitric Oxide/ Cyclic Guanosine Monophosphate (cGMP)/ROS Balance in the TGFβ1 Rat Model of PD and Applications of This Model

An alternative view on the role of iNOS spontaneous induction in the TGFβ1-elicited PD-like plaque in the rat was proposed in 2002 by Ferrini et al. from UCLA after the authors noticed increased fibrosis and plaque formation in the penis when a specific inhibitor of iNOS, L-N-(1 iminoethyl) lysine acetate (L-NIL), was given to the animals for six weeks following the TGFβ1 injection into the tunica albuginea. From this observation, they proposed that iNOS operates as an antifibrotic factor in this setting [23]. The hypothesis that iNOS is antifibrotic is consistent with what has been observed in other tissues, such as the heart, liver and kidney, when fibrosis develops after these tissues have been exposed to both long-term and continuous inhibition of total nitric oxide production by a general NOS inhibitor such as Nomega-nitro-L-arginine methyl ester (L-NAME).

The first assumption of Ferrini's article was that nitric oxide was able to bind to ROS, the profibrotic compounds produced by oxidative stress, in a reaction that produces peroxynitrite. This is an apoptotic but presumably nonfibrotic compound. Nitric oxide produced from NOS isoforms would actually quench ROS, which, if left undisturbed, would elicit and maintain PD plaque development. The second assumption was that this nitric oxide did not originate from nNOS or eNOS, which are localized in the corpora cavernosa nerves and endothelium and are modulated at the enzyme

activity level by neurotransmission or hemodynamic processes. The source of this nitric oxide would be the iNOS induced within the tunical fibroblasts via transcriptional activation by cytokines. In such a setting, iNOS can produce steady levels of nitric oxide, which can then act as an endogenous defense mechanism against fibrosis. This article showed that in the human PD plaque, as compared with normal tunica, iNOS mRNA and protein were both induced. This induction occurred in parallel to collagen deposition that was estimated histochemically and by hydroxyproline levels. Both oxidative stress, as measured by hemoxygenase-I, and nitric oxide/ROS reaction, as measured by nitrotyrosinilation of proteins, accompanied these processes.

To test the role of iNOS induction in this experimental setting, the specific inhibitor of iNOS enzyme activity, L-NIL, was given in the drinking water continuously for 45 days to rats injected into the tunica with either TGFβ1 to produce the plaque or with saline as a control. It was determined that collagen deposition and oxidative stress were dramatically increased while peroxynitrite was reduced in the TGFβ1-injected tunica in comparison with saline-injected controls. This iNOS induction in the human plaque as well as the results of the L-NIL experiments suggested that iNOS was acting as a defense mechanism against fibrosis.

Further proof of the effect of iNOS was obtained when in both the human PD plaque and the TGFβ1 PD-like plaque in the rat model, myofibroblast formation was shown to have considerably increased. This was denoted by the cells expressing α-smooth muscle actin (*ASMA*), a marker of both myofibroblasts and smooth muscle cells, as compared with the ones expressing vimentin, solely a fibroblast marker [24]. Myofibrobast formation occurred in tandem with an increase in collagen synthesis, as detected by the activation of the collagen Iα gene promoter in a recombinant DNA construct. This promoter directs the expression of β-galactosidase as a reporter protein, which is very easy to detect histochemically, and was injected into the tunica albuginea of the saline or TGFβ1-injected rats 1 week before sacrifice. As expected, chronic administration of L-NIL intensified collagen synthesis, which paralleled the amount of ASMA expression.

Moreover, cell culture experiments based on the incubation of fibroblast cultures from the human PD plaque and the normal tunica albuginea using L-NIL, and cGMP and nitric oxide donors, confirmed these results. Although myofibroblasts were previously detected in the human PD plaque [13], this was the first demonstration in vivo and in vitro, and in the human PD and PD-like plaque in the rat, that myofibroblasts differentiated from normal tunica albuginea fibroblasts and increased during plaque formation. Moreover, this showed that both cGMP and nitric oxide counteracted myofibroblast accumulation. Myofibroblasts are key cells during wound healing, which, at the completion of this process, are normally eliminated by apoptosis. When they persist, this is abnormal, and such persistence leads to scar formation [25]. Moreover, their accumulation in normal tissues is, together with collagen deposition and oxidative stress, a landmark of the development of fibrosis.

These observations led to the natural conclusion that a sustained pharmacological increase of cGMP and/or nitric oxide by a long-term continuous administration of drugs such as the PDE5 inhibitors and/or nitric oxide generators, should reduce the fibrotic plaque in the TGFβ1 rat model of PD. Two subsequent articles from the UCLA group supported such a hypothesis. In the first one [26], sildenafil (as a cGMP-dependent PDE5 inhibitor), L-arginine (as a NOS substrate), and pentoxifylline (as a nonspecific cAMP-dependent PDE inhibitor) were given separately to three groups of animals in the drinking water for the 45 days following the induction of a plaque in the tunica albuginea by TGFβ1. All three compounds prevented the appearance of the PD-like plaque when assessed by collagen deposition, myofibroblast formation, and apoptosis of myofibroblasts. This was confirmed in vitro in the cell cultures from the human tunica albuginea and PD plaque, in parallel to the expected increase of cGMP or cAMP levels. Moreover, phosphodiesterase type 5 (sildenafil target) and PDE4 (pentoxifylline target) were found to be expressed in both the human and rat tunical and PD tissues and in their respective cell cultures. To our knowledge, this was the first experimental demonstration of an antifibrotic effect for PDE5 inhibitors.

Additional similar studies were performed with another PDE5 inhibitor, vardenafil, where the drug was given not only in a continuous long-term way in the drinking water as was done with sildenafil, but also in a more natural way by daily single retrolingual administration [27]. With this daily

form of administration, the plaque was similarly inhibited. Moreover, in this study, it was shown for the first time that partial regression of an already formed plaque could occur when vardenafil is given in high doses for 14 consecutive days, beginning once the plaque has already formed.

Besides these two aforementioned PD studies, our group has provided additional experimental evidence to support the view that PDE5 inhibitors administered on a long-term treatment do indeed posses antifibrotic properties. One study involved the demonstration that the normal aging related fibrotic changes in the corporal tissue can be ameliorated by these PDE5 inhibitors, while the second series of experiments involved the reduction of fibrosis of the corpora that follows cavernosal nerve injury [28]. In this latter model of cavernosal fibrosis, PDE5 inhibitors prevented the onset of cavernosal fibrosis following cavernosal nerve injury [29–31].

The TGFβ1-induced rat PD model also served to provide the initial insight into the role of another member of the TGFβ family, myostatin, which was identified in the human PD plaque [32]. Myostatin or GDF-8 (growth and differentiation factor 8) is a negative regulator of skeletal muscle mass in the human and other animals [33]. It modulates the entry of multipotent stem cells into myogenesis and adipogenesis [34], and it stimulates the development of the fibrotic phenotype of myofibroblasts from these multipotent cells [35]. The profibrotic effects of myostatin were confirmed by showing that a cDNA expressing this protein induced per se a PD plaque in the tunica albuginea [32] of the rodent, similar to that seen with TGFβ1. Although both myostatin and TGFB1 signal through a common Smad pathway, they appear to act independently in causing tunical fibrosis in the rat model of PD. This suggests that therapeutic inhibition of the Smad pathway, or common binding of both myostatin and TGFβ1 by agents like decorin, may be an effective way of controlling the progression of PD.

A recent improvement of the TGFβ1 rat model of PD appears to have reduced two of its major shortcomings, i.e., the absence of penile curvature, one of the main features of PD in men, as well as a calcification/ossification process that is seen in 15–25% of PD patients [36]. This was based on the administration of multiple injections (at 0, 3, and 6 days) of a "low" dose

(10^{10} particles) of a replication-deficient adenoviral construct expressing the full-length TGFβ1 protein, which, rather than just a standard single TGFβ1 injection, or a single injection of the TGFβ1 construct at higher doses, or a single injection of the recombinant TGFβ1 protein, caused a modest but evident 20-degree bending of the rat penis at 6 weeks, and no cartilage formation [37]. Unfortunately, no specific markers of ossification, such as alkaline phosphatase or von Kossa, were studied by these investigators. A similar construct expressing reporter protein β-galactosidase instead of TGFβ1 did not cause any tunical plaque, curvature, or cartilage/bone formation. Moreover, the plaque trapped inflammatory cells in the tunica and the loss of elastin fibers, although the diagnostic marker TGFβ1 was not measured, and the curvature (but not the plaque) disappeared spontaneously at 60 days. Interestingly, in this model, the plaque that was sufficient to induce penile curvature did not, however, lead to the erectile dysfunction, which had been claimed in the earlier work with TGFβ1 protein even in the absence of any curvature [18].

The Fibrin-Induced Model of PD and the Microtrauma Hypothesis

As mentioned above, early articles had proposed that PD was caused by an abnormal wound healing following some form of trauma to the erect penis, thus equating the plaque with scar tissue [7,8]. It was assumed that blood proteins, among them fibrinogen, would be released from the injury into the fractured tunica albuginea and then be converted into fibrin. Persistence of this fibrin by an abnormal inhibition of the fibrinolytic system would trigger initially an acute, and later, a chronic inflammatory response, with the subsequent production of TGFβ1, reactive oxygen species, and other profibrotic factors that would then induce the development of the fibrotic plaque that characterizes PD.

In an attempt to mimic this process in an animal model, Davila et al. injected a special fibrin preparation into the rat tunica albuginea and compared this group with animals that were injected with TGFβ1 alone, with saline used as a control group, and then a group injected with both fibrin and TGFβ1 [11]. A fibrotic plaque was induced by the fibrin injection as early as 3 weeks (half of the time required for plaque formation with TGFβ1 injec-

tion). Fibrosis in this fibrin model was accompanied by the hallmarks of the TGFβ1-induced plaque, namely TGFβ1 expression, oxidative stress, myofibroblast formation, iNOS induction, peroxynitrite formation and, induction of plasminogen activator inhibitor 1 (*PAI-1*), a strong indicator that inhibition of fibrinolysis was occurring. Indeed, when the human PD plaque was then analyzed for the presence of fibrin and PAI-1, both were identified in the human tissue, thereby lending credence to the theory that the fibrin-induced PD-like lesion in the rat tunica albuginea was a potential model for the sequence of events occurring in the development of human PD.

Confirming the assumption that iNOS acts in this fibrin model as an antifibrotic agent in the same fashion as it is postulated to function in the TGFβ1 model, gene therapy with a plasmid construct of the iNOS cDNA injected in the tunica albuginea of rats induced regression of the PD-like plaque that was induced with fibrin [38]. In addition, treatment with the iNOS cDNA was also associated with a reduction in the expression of profibrotic factors and oxidative stress markers.

Cell Culture Models of PD and Tunica Albuginea Stem Cells

Although the rat models have been pivotal in understanding the molecular and cellular pathology of PD, cell cultures from the human PD plaque and the normal tunica albuginea have also provided very valuable information, particularly in terms of myofibroblast differentiation, gene expression, and the role of tunical stem cells. After the initial description of myofibroblasts in the plaque and the isolation of the first cell cultures from this tissue [12], no further studies were conducted until 2000, when different cell cultures were characterized from the plaques of several PD patients and from normal human tunica albuginea [13]. The cells of the normal tunica albuginea were shown to be primarily fibroblasts. In the PD cell cultures, myofibroblasts, as well as fibroblasts, were present, and these cells demonstrated cytogenetic instability, excess production of fibrogenic cytokines, and other functional alterations [39–41]. It was proposed that PD cell cultures could be useful to devise new therapeutic strategies by trying to prevent these alterations.

In fact, PD and tunical cell cultures were used in an already cited study [24] to investigate the effect of nitric oxide and cGMP on collagen synthesis, on the presence of PDE5 and PDE4 in these cells [26], and on the up regulation of monocyte chemoattractant protein 1 (*MCP1*) in PD cells as compared with normal tunical cells [42]. MCP1 overexpression had been also characterized by DNA microarrays and confirmed by RT/PCR and western blot in human plaque tissue. These DNA microarrays also showed overexpression of other cytokines, collagen synthesis, markers of inflammation, fibroblast proliferation, and myofibroblast and osteoblast differentiation [43,44].

This was followed up by the comparison of multiple gene expression in the respective cells and tissues from both human PD and normal tunica albuginea, and in Dupuytren's nodules, a condition associated with PD [45]. The pattern of alterations of gene expression was common for certain gene families for the PD plaque tissue and cultured cells as compared with the Dupuytren's tissues, using as controls the respective normal tissues and cells. These features were essentially the upregulation of markers of myofibroblast and osteoblast differentiation as indicators of fibrosis and calcification, and paradoxically of collagen degradation (metalloproteinases 2 and 9 (MMP 2 and 9), thymosins), and of decorin, an anti-TGFβ1 factor. This would suggest that in PD tissue, there appears to be activation of an endogenous antifibrotic mechanism additional to iNOS expression, as well as the existence of active tissue turnover. The presence of tissue turnover within a scar as was seen in the PD tissue implied that the PD plaque composition was not an irreversible end point and that it may be susceptible to modulation by pharmacological intervention targeting the balance between several processes. These include at least: (i) the nitrosative reaction and oxidative stress; (ii) the TGFβ family signaling/decorin interaction; and (iii) the collagen synthesis/degradation balance. In other words, it showed that the PD plaque, as well as the Dupuytren's nodules, is in a state of flux that potentially can be pushed toward a decrease in fibrosis by stimulating or upregulating the respective endogenous defense processes.

In addition to fibrogenic cytokine expression [46,47], the PD tissue has another feature characterized in the PD cell model—namely, the fact that these cells are potentially tumorigenic or acquire this trait upon culture. This was shown by Mulhall et al. [9], who implanted these cells into SCID mice and found that subcutaneous tumors devel-

oped in all animals, whereas none occurred with fibroblast cultures from the normal tunica albuginea or foreskin [48,49]. Cultures similar to those of the human PD plaque tissue were also obtained from the rat tunica albuginea and the PD-like plaque induced by TGFβ1, but they were not tested for tumorigenesis [26].

The tumorigenic feature of PD cells was confirmed by Vernet et al. [50] by selection in soft agar, a procedure used to detect either cancer cells or hematopoietic stem cells. It was shown that these cultures express CD34 (stem cell marker), and upon incubation with TGFβ1, undergo differentiation into skeletal myofibroblasts, smooth muscle cells, and osteoblasts, but not adipocytes. In addition, these tunical cells could paracrinely modulate in dual cell culture the differentiation of a multipotent cell line into osteoblasts and myofibroblasts. The presence of stem cells in the normal tunica albuginea may explain the fibrotic and osteogenic progression of the PD plaque upon the release of cytokines following microtrauma to the penis, which would stimulate this cell lineage commitment. Since stem cells can replicate indefinitely, they may lose the control of cell replication in certain tissue environments or by a long time in culture, and thus become tumorigenic, but why this does not occur in PD remains to be determined.

Cells expressing the stem cell markers CD34 and Sca1 were also found in vivo in the normal penile tunica albuginea, as well as in the corpora cavernosa [51]. They were identified as potential endogenous stem cells, since they are likely the ones that, in the in vitro models, undergo multiple lineage differentiation, and in the PD plaque, may convert first into myofibroblasts, and, later, into osteoblasts. These stem cells may also provide a target for pharmacological therapy of PD aimed to block their role as sources for the de novo formation of the differentiated cells (myofibroblasts, osteoblasts) that cause fibrosis and ectopic ossification. In fact, the fibrotic commitment of these multipotent cells may possibly be inhibited by long-term continuous administration of PDE5 inhibitors in vivo, as discussed above. This cell culture model actually showed that a similar long-term treatment in vitro (with the PDE5 inhibitor, tadalafil) did not upregulate PDE5, thus reducing the possibility that tachyphylaxis would be an impediment for their potential daily use for long periods of time to reverse fibrosis [52].

It has recently been shown that TGFβ1 induces the Smad pathway, the common signaling for the profibrotic effects of TGFβ family members. Interferon gamma, which has been used clinically to treat PD, did not by itself abrogate the Smad pathway, but when given together with TGFβ1, the Smad signaling was stimulated. The authors concluded that interferon gamma may not be useful for PD treatment [53]. However, this interpretation must be taken with caution, since the authors did not address the possibility of interferon gamma stimulating antifibrotic pathways, like the effect that would stem from its well-known iNOS induction activity.

A recent article using tunical fibroblast cultures [54] has served to emphasize the role that TGFβ has in strongly inducing tissue inhibitors of metalloproteinases (TIMPs), well-known inhibitors of MMP, without affecting MMPs themselves, and hence in interfering with the breakdown of collagen fibers deposited in excess during the development of the PD plaque. In contrast, interleukin-1β strongly induces MMP 1, 3, 10, and 13 expression. These findings may help to explain why the overexpression of MMP 2 and 9 mRNAs previously detected in the PD plaque [45] and assumed to be an endogenous defense mechanism against fibrosis, does not translate into an intensified collagenase activity. TIMPs induced by TGFβ and other profibrotic factors may counteract this defense mechanism.

The latter article also brings to the forefront the MMP/TIMP/PAI-1 system, an interplay of collagenolytic and fibrinolytic pathways and their inhibitors, which seems to be deeply altered in PD, and the need to reexamine the potential value of specific MMP administration for the therapy of PD, or the pharmacological modulation of key factors in this interaction. The inhibition of the fibrinolytic system by high PAI-1 levels [55] may contribute to the persistence of fibrin, already shown to cause a PD-like plaque in the animal model [11], elicit TGFβ1, and increase collagen. Normal collagen content cannot then be maintained through its breakdown by MMP, because MMP activity would be reduced by high levels of TIMPs.

Conclusions

The development of the first animal model of PD based on TGFβ1 injection into the tunica albug-

inea, and the further characterization of the PD cell cultures, initiated a decade of significant contributions to the knowledge of the molecular and cellular pathology of PD. In a way, these models had an impact on PD research similar to the one exerted by animal models of erectile dysfunction on the elucidation of the basic pathophysiological mechanisms underlying this disorder [56]. Collectively, they helped to: (i) to clarify the role of microtrauma, cytokines, myofibroblasts, and oxidative stress in plaque initiation and progression; (ii) to demonstrate that this tissue is under sustained turnover as a result of sustained fibrotic and antifibrotic mechanisms; (iii) to emphasize the interplay of the collagenolytic and fibrinolytic systems and their inhibitors in this turnover; (iv) to show that one endogenous antifibrotic process is the expression of *iNOS*, which results in nitric oxide and cGMP formation in order to inhibit oxidative stress, collagen synthesis, and myofibroblast generation; (v) to characterize the antifibrotic effects of PDE5 inhibitors given on a long term basis in order to mimic the endogenous process; (vi) the discovery of the cytogenetic instability of the PD cells and alterations in their gene expression; and (vii) the finding of stem cells in the tunica albuginea with a potential role in fibrosis and ossification.

In synthesis, the two experimental rat models share some of the histological features that were defined in the human PD plaque (e.g., [23]), such as initial inflammation and subsequent excessive deposition and disorganization of collagen fibers, elastin fragmentation, accumulation of myofibroblasts and profibrotic factors (oxidative stress, PAI-1, TGF β1, and fibrin), and of potential antifibrotic factors (iNOS), and eventual calcification and ossification. However, as it occurs with most animal models, they cannot truly represent the complexity of the human disease. Perhaps a major concern is that, sometimes, tunical fibrosis is accompanied by a similar process in the adjacent corporal tissue, although this likely results from the technical difficulty of limiting the injection exclusively to the tunical tissue. Further work may be needed in larger animals to reduce this risk. Similarly, although cell cultures from the human plaque and normal tunica contain the myofibroblasts, fibroblasts, and stem cells that in vivo participate in the fibrotic process, they cannot mimic the interplay of paracrine and juxtacrine factors that operate in vivo, including the cross-talk with corporal cells.

Table 1 Novel pharmacological strategies for Peyronie's disease

Basic premise: shift tissue turnover to:
• Predominance of collagen breakdown over synthesis
• Predominance of myofibroblast apoptosis over proliferation
• Switch off myofibroblast and osteoblast differentation from stem cells

However, even with the limitations intrinsic to experimental vs. clinical research, these models may be improved and even used in combination to develop novel long-term pharmacological approaches to reduce the PD plaque. Just to name a few strategies, the inhibition of myofibroblast differentiation from endogenous stem cells, the stimulation of myofibroblast apoptosis, the reduction of collagen cross-linking, the induction of selective MMPs in conjunction with anti PAI-1 agents, or the combination of PDE5 inhibitors with anti-inflammatory agents (Tables 1 and 2), are potentially approachable in both the animal and cell models. They may well serve to extrapolate to PD some promising experimental studies on the molecular pathology of tissue fibrosis in general aimed to counteract this process in such organs as the liver, kidney, lung, or skin, where there is ongoing intense research activity.

Even if PD is not a life-threatening situation, the combination of its relatively high prevalence

Table 2 Promising pharmacological targets for Peyronie's disease

Excessive collagen deposition
• NO donors and PDE5 inhibitors (increase NO/cGMP, inhibit collagen synthesis).
• Decorin/follistatin (counteract TGF(/myostatin).
• Smad 7 and related (counteract downstream Smad pathway for TGF(family).
• β-thymosins (reduce collagen synthesis; promote healing).
• Pirfenidone and other antifibrotic agents (anti-inflammatory, reduce cytokines and oxidative stress).
• Antioxidants (reduce ROS and oxidative stress).
• MMP/anti-TIMP (increase collagen degradation).
• Endogenous MMP upregulation, activation (increase collagen degradation).
• Collagen cross-link breakers, Alt 711 and similar (facilitate collagen degradation).

Myofibroblast accumulation
• NO donors and PDE5 inhibitors (reduce fibroblast/stem cell differentiation, increase apoptosis).
• New agents targeting stem cell commitment to myofibroblast formation or syntetic phenotypic switch.

NO = nitric oxide; PDE5 = phosphodiesterase type 5; cGMP = cyclic guanosine monophosphate; TGF(1 = transforming growth factor (1; ROS = reactive oxygen species; MMP = metalloproteinase; TIMP = tissue inhibitor of metalloproteinases.

and its impact on quality of life and public health costs, should be a call of attention for sponsoring more basic research, particularly considering the applicability of these experimental models to the elucidation of important issues in the general mechanisms of fibrotic and antifibrotic processes in a variety of diseases.

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Mechanisms of Penile Fibrosis

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ABSTRACT

Introduction. Penile fibrosis has been conceptually identified with the plaque that develops in the tunica albuginea in Peyronie's disease (PD), or with localized processes induced in the corpora cavernosa by ischemic or traumatic events. Recently, it has been proposed that a diffuse, progressive, and milder intracorporal fibrosis, which affects also the media of the penile arteries, is responsible for vasculogenic erectile dysfunction (ED) associated with aging, smoking, diabetes, hypertension, and post-radical prostatectomy. These processes differ in etiology, time course, target cells, and treatment, but have many features in common.
Aim. To review the literature pertaining to fibrosis in the penis, related to PD and ED.
Methods. PubMed search for pertinent publications mainly during 2001–2008.
Results. This review focuses initially on PD and then deals with studies on ED in animal and cell culture models, discussing some of the pathophysiological similarities between tunical fibrosis in PD and corporal fibrosis in corporal veno-occlusive dysfunction (CVOD), and emerging therapeutic strategies. The role of profibrotic factors, the excessive deposit of collagen fibers and other extracellular matrix, the appearance of a synthetic cell phenotype in smooth muscle cells or the onset of a fibroblast–myofibroblast transition, and in the case of the corporal or penile arterial tissue the reduction of the smooth muscle cellular compartment, are discussed. This histopathology leads either to localized plaques or nodules in penile tissues, or to the diffuse fibrosis causing impairment of tissue compliance that underlies CVOD and arteriogenic ED. The antifibrotic role of the sustained stimulation of the nitric oxide/cyclic guanosine monophosphate pathway in the penis and its possible relevance to exogenous and endogenous stem cell differentiation is also briefly presented.
Conclusions. Fibrotic processes in penile tissues share a similar cellular and molecular pathophysiology and common endogenous mechanisms of defense that have inspired novel pharmacological experimental approaches. **Gonzalez-Cadavid NF. Mechanisms of penile fibrosis. J Sex Med 2009;6(suppl 3):353–362.**

Key Words. Erectile Dysfunction; Corporal Veno-Occlusive Dysfunction; Inducible Nitric Oxide Synthase

Introduction

The topic of urogenital fibrosis is dominated by the considerable significance of tubulointerstitial fibrosis and glomerulosclerosis in chronic kidney disease, mainly diabetic nephropathy [1,2], and of postsurgical adherences [3], but very little attention has been focused on fibrotic processes in other urogenital disorders. Until recently, penile fibrosis was assumed to be limited to the Peyronie's disease (PD) plaque in the tunica albuginea [4] or to the comparatively rare events subsequent to tissue insults such as intracorporeal injection or prolonged priapism [5]. However, in the last few years, it has become evident that fibrosis of the corpora cavernosa and the media of penile arteries, involving loss of smooth muscle cells (SMC), is a highly prevalent process that underlies most cases of vasculogenic erectile dysfunction (ED) (see e.g., [6–10]). Therefore, the study of fibrosis may provide a unifying view on the two most prevalent disorders affecting the penis, even if located in different tissues. This presentation discusses some selected results, focusing on the contributions from our group at the University of California, Los Angeles.

- **Excessive accumulation and disorganization of collagen fibers and other extracellular matrix, with reduction of cell number and, usually, myofibroblast accumulation**
- **Diffuse or localized in most organs; also in abnormal wound healing**
- **Leads to tissue and organ dysfunction**
- **Subsequent to acute injury or chronic inflammation, with release of cytokines (transforming growth factor beta 1), plasminogen activator inhibitor 1, and oxidative stress (reactive oxygen species)**

Figure 1 Main features of the fibrotic process.

The excessive deposition of collagen and extracellular matrix (ECM) accompanied by the loss of functional cells that characterize tissue fibrosis, is due in some cases to the appearance and accumulation of myofibroblasts or in other cases to the switch to a synthetic phenotype producing ECM of the original cell components, such as fibroblasts and/or SMC in the penis (Figure 1). The main factor in eliciting these cellular alterations is an insult to the tissue, be it: (i) acute and localized, in a specific site in the tunica albuginea in PD [11,12]; (ii) acute and diffuse throughout the corpora such as in cavernosal nerve damage after radical prostatectomy [8,13–19]; or (iii) chronic and also diffuse throughout the corpora and the penile arteries wall such as in aging, diabetes, and heavy smoking [9,10,20–26]. The corporal and arterial alterations lead to corporal veno-occlusive dysfunction (CVOD), the most prevalent form of vasculogenic ED [27] that can be measured in the rat by cavernosometry [28]. The initial insult to any of these penile tissues results in the release of profibrotic factors, mainly transforming growth factor beta 1 (TGFβ1), plasminogen activator inhibitor 1 (PAI-1), and reactive oxygen species (ROS) leading to oxidative stress, that may be in some cases exacerbated by chronic inflammation. This is remarkably similar to what occurs in the more widely studied diffuse fibrosis in kidney, lung, liver, and skin, or the localized processes in abnormal wound healing leading to scars or myocardial infarction [1,29–33].

Fibrosis of the Tunica Albuginea in Peyronie's Disease

The main culprit of fibrosis in PD is the myofibroblast, that is key for normal wound healing but is eliminated by apoptosis after the tissue is healed;

when this does not occur, fibrosis develops [33]. It is likely that the same occurs in vascular and corporal fibrosis, but the difficulties in differentiating myofibroblasts from SMC have not yet allowed their proper identification. Experimental studies in PD were based in the combination of two human models (the PD plaque and its normal tunical counterpart, and cell cultures derived from these tissues) and two animal models where the lesion was elicited by either TGFβ1 or fibrin [34] (Figure 2). They, together with those of Mulhall's group [35,36], have shown the appearance and persistence of myofibroblasts in PD and have clarified some of their features.

In those earlier studies, we showed that oxidative stress and PAI-1 increase in the PD plaque, and very important, that the inducible nitric oxide synthase (iNOS) isoform that produces nitric oxide (NO) and is usually considered a defense mechanism against infection or cancer and is associated with inflammation, is remarkably increased in the human and animal plaques [11,37–43]. This was illustrated by the simultaneous increase of iNOS and an antioxidant enzyme in the fibroblasts of the human PD plaque evaluated by immunohistochemistry/quantitative image analysis (QIA) and by the reduction of the PD-like plaque stained for collagen by Masson trichrome and QIA. Moreover, the plaques are not an irreversible dead-end but are in a constant state of molecular and cellular turnover, as determined by DNA microarrays, involving a balance between fibrotic and antifibrotic mechanisms [44,45] (Figure 3).

The accumulation of myofibroblasts in the fibrotic PD plaque was detected, as compared with the normal tunica albuginea, by immunohistochemical staining and quantitative image analysis for α-smooth muscle actin (ASMA), a marker of both myofibroblasts and SMC, and also by Western blot. Cell cultures followed by both types of assays confirmed these findings [35,36,39]. The spontaneous chronic induction of iNOS, a leitmo-

- **PD plaque and tunica albuginea tissues in men**
- **Fibroblast cultures from tissues**
- **First rat model (1997, UCSF): PD-like plaque in the rat tunica by transforming growth factor beta 1 (TGFβ1) peptide**
- **Second rat model (2003, UCLA): fibrin injection in the rat tunica, eliciting TGFβ1**

Figure 2 Models for studying fibrosis in Peyronie's disease (PD).

- **Excessive deposition of collagen fibers, oxidative stress, and persistence of myofibroblasts, seen in other fibrosis (2002-3, UCLA)**

- **Spontaneous expression of inducible nitric oxide synthase leading to a sustained output of nitric oxide protecting against oxidative stress and fibrosis (UCLA, 2002-3)**

- **The PD plaque is under a steady cellular and molecular turnover (2003-5, UCLA)**

Figure 3 The PD plaque as a model of localized penile fibrosis.

tif of other fibrosis, was postulated by us to be an antifibrotic mechanism, based essentially on the exacerbation of fibrosis, myofibroblast production, and oxidative stress in the rat PD-like plaques by blockade of iNOS activity with L-N-(1-iminoethyl)-lysine acetate (L-NIL), and conversely of their reduction by gene transfer of iNOS complementary DNA (cDNA) to the plaque [38,39,42] (Figure 4).

This led us to postulate that the steady production of NO from iNOS quenches ROS, producing peroxynitrite that reduces the levels of profibrotic ROS, and on the other hand NO from iNOS causes myofibroblast apoptosis. NO, as well as its product, cyclic guanosine monophosphate (cGMP), also inhibits collagen synthesis directly as demonstrated in fibroblast cultures from the normal human tunica [39]. The pharmacological implication was that phosphodiesterase type 5 (PDE5) inhibitors, by elevating the content of cGMP in penile tissues, would reverse the plaque and combat myofibroblast accumulation. First with sildenafil, and later with vardenafil given from the induction of the plaque and even after it was formed, we showed by immunocytochemistry/QIA that ASMA staining was considerably reduced, as well as plaque size and oxidative stress [46–48].

TGFβ1 may be the main, but is not the only profibrotic factor involved in the development

- **iNOS spontaneous induction in the rat PD-like plaque as in the human PD (2000, Tulane; 2002, UCLA)**

- **iNOS inhibition in the rat models increases fibrosis, myofibroblasts, and oxidative stress (2002-3, UCLA)**

- **iNOS induction by gene transfer inhibits these processes (2004-5, UCLA)**

Figure 4 Inducible nitric oxide synthase (iNOS) is an antifibrotic factor in Peyronie's disease (PD).

Basic premise: shift tissue turnover to:

- **predominance of collagen breakdown over synthesis**

- **predominance of myofibroblast apoptosis over proliferation**

- **switch off myofibroblast and osteoblast differentiation from stem cells**

Figure 5 Novel pharmacological strategies for PD.

of the plaque. Very recently we showed by immunohistochemistry/QIA that another member of this superfamily that comprises activins, myostatin or GDF-8, is considerably expressed in the human PD plaque, mainly in myofibroblasts [49]. By using cDNA for myostatin, we demonstrated in the rat model that myostatin per se induced a plaque or exacerbated the one produced by TGFβ1 as shown by Masson trichrome/QIA. However, this effect was ancillary to the action of TGFβ1 and not essential, as blocking myostatin production with a small hairpin RNA (shRNA) against this protein did not affect the size of the TGFβ1-induced plaque.

These studies, and particularly the concept that fibrosis is not irreversible, that the key target cell is the myofibroblast, and that it appears to originate from stem cells that are present in the tunica albuginea and originate other cell lines, justify to investigate novel strategies for the pharmacological therapy of PD to block these processes, particularly the modulation of stem cell or progenitor cells in the tunica [25,43] (Figure 5).

Fibrosis of the Corporal Tissue and Arterial Media Associated with Vasculogenic ED

As stated initially, fibrosis of the corporal smooth muscle and the penile arteries media has emerged as the predominant underlying cause of ED caused by the most diverse risk factors [5–10,13–26] (Figure 6). iNOS plays here the same antifibrotic role occurring in PD, and even in the diabetic vagina [50] that can be exploited pharmacologically with similar approaches to the ones used for PD [51]. This is shown by the fact that corporal fibrosis measured by Masson trichrome/QIA for the SMC/collagen ratio (the inverse ratio of the one used for PD where collagen/extracellular ratio was measured) and by hydroxyproline determinations in penile tissue hydrolyzates, presents during aging of the iNOS knockout (ko) mouse where iNOS expression is genetically abrogated, already in the young adult stage (8 months of age) [52].

- **Most common form of ED is corporal veno-occlusive dysfunction (CVOD)**
- **Associated with aging, diabetes, cavernosal nerve damage, and possibly low testosterone**
- **CVOD is caused by fibrosis of the smooth muscle of the corpora cavernosa and the media of the penile arteries**
- **Inducible nitric oxide synthase, as in Peyronie's disease, plays an antifibrotic role that can be mimicked pharmacologically**

Figure 6 The predominant underlying cause of erectile dysfunction (ED) is diffuse corporal tissue fibrosis.

This continues throughout the life span, whereas in the wild type animal there is a progressive but mild fibrosis peaking at 20 months of age. A similar exacerbation of fibrosis by iNOS deletion is seen in the iNOS ko mouse rendered diabetic by streptozotocin injection [53].

The corporal fibrosis, denoted in the trabecular tissue of the corpora containing the SMC, by the decrease of the SMC/collagen ratio and of the ASMA positive staining, is also evident in the experimental rat, specifically in the models for aging, diabetes, and castration as compared with their respective controls [9,20–23,25,28]. These histological alterations underlie CVOD, as measured by dynamic infusion cavernosometry particularly in terms of the drop rate.

As in the case of PD, long-term continuous treatment for 2 months of aged rats (20-month old) with an oral PDE5 inhibitor, in this case sildenafil, in the drinking water corrects CVOD as shown by cavernosometry after a 3-day washout [9], and the same occurs with a peroxisome proliferator activated receptor γ (PPARγ) agonist, pioglitazone, that presumably acts via an antioxidant and anti-inflammatory mechanism on both aged and diabetic rats [22,23]. This is accompanied, as expected, by a substantial increase of corporal SMC determined by ASMA immunohistochemistry/QIA.

The CVOD occurs also in adult rats after bilateral cavernosal nerve resection (BCNR) that mimics the cavernosal nerve damage in men after non-nerve sparing radical prostatectomy, and this develops gradually as a consequence of the neuropraxia, with is first manifestation seen between 15 and 30 days after BCNR. This functional alteration is preceded by a significant decrease in the SMC/collagen ratio or the SMC content in the corpora at 7 and 3 days, respectively, after BCNR [8,16–19]. The loss of SMC appears to result from

a predominance of apoptosis, determined by the TUNEL procedure, over the initial bout of cell proliferation, measured by immunohistochemistry for proliferating cell nuclear antigen, that aims to counteract the apoptosis already seen as early as 1 day after BCNR [19]. There is a sustained increase of iNOS that peaks at 30 days.

Again, as in the case of the aged rat and PD, the three PDE5 inhibitors, tadalafil, sildenafil, and vardenafil given as a sustained oral administration for 45 days immediately after BCNR prevent CVOD, while restoring the normal SMC/collagen ratio estimated by Masson/QIA to about the same extent [8,16–18]. These results have been replicated using a milder nerve injury procedure in the cavernosal nerve crush injury model, where a daily treatment with sildenafil for 28 days resulted in the protection of the SMC/collagen ratio and the endothelium, and the improvement of the electrical field stimulation (EFS) response in a time- and dose-dependent fashion [54]. Interestingly, the three PDE5 inhibitors administered orally for short periods (up to 36 hours) to normal intact rats also increased heme oxygenase activity, which would suggest that they may reduce oxidative stress and hence pro-fibrotic ROS, but this has not yet been tested in any of the corporal fibrosis models [55].

The preservation of the SMC content in the corpora may also be exerted by antiapoptotic effects of other agents such as growth differentiation factor 5 (GDF-5) or the immunophilin ligand FK506 in the cavernosal nerve injury model [56,57]. Similarly, insulin-like growth factor 1 (IGF-1) given to aged rats for 4 or 8 weeks increased the percentage of corporal SMC and stimulated the erectile response to EFS [58].

The SMC fibrosis is not restricted to the corpora, because during aging it is also seen in the media of the penile arteries, in this case the penile dorsal artery (PDA) [10]. iNOS is also overexpressed in the aged arteries and its blockade leads to an increase in fibrosis measured by SMC/collagen ratio. An identical loss of SMC and increase in apoptosis occurs in the PDA and the aorta in the ZDF rat, a model for type 2 diabetes [24].

Integrated View of Fibrosis Mechanisms in Penile Tissues

Figure 7 compiles these results and shows that in the tunica adventitia, the corpora cavernosa, and the penile arterial media, the decrease of the cellular/collagen ratio, and the increase of total

Tissue	Species	Condit	Cell/Coll	Coll	Myofib	ASMA
• Tunica	Human	PD	↙	↗	↗	↗
	Fisher rat	"	↙	↗	↗	↗
• Trabec	" "	Aging	↙	↗	?	↙
	iNOS ko	"	↙		?	↙
	ZDFfa/fa	Diab 2	↙	↗	?	↙
	Fischer rat	Diab 1	↙		?	↙
	iNOS ko	"	↙		?	↙
	Fischer rat	BCNR	↙	↗	?	↙
• PDA	" "	Aging	↙		?	↙
	Zucker fa/fa	Diab II	↙		?	↙

Figure 7 Cellular/extracellular matrix balance in penile fibrosis.

ASMA = α-smooth muscle actin; BCNR = bilateral cavernosal nerve resection; C = collagen; Condit = condition; Diab = diabetes; Myofib = myofibroblast; iNOS = inducible nitric oxide synthase; PD = Peyronie's disease; PDA = penile dorsal artery; Trabec = corporal trabecular tissue.

collagen when this was measured, are the common denominators, irrespective of the type of tissue, the animal model, or the pathological outcome. ASMA, a dual myofibroblast/SMC marker, is increased when there is myofibroblast accumulation in the tunica in PD, but reduced when the SMC are lost in the corpora or the arteries in the models of CVOD. However, because of the duality of ASMA as a marker, no information is as yet available on the presence of myofibroblasts in the corporal or arterial smooth muscle, as they cannot be discriminated with ASMA from SMC. Figure 8, in turn, shows iNOS induction to be the other

common denominator, whereas apoptosis is seen in all cases of corporal fibrosis. On the other hand, TGFβ1 induction is restricted to PD or the vagina [11,38,50] as it was not seen as a significant process in the penile corpora cavernosa during aging, diabetes, or BCNR. Oxidative stress occurs mainly in PD [11,38] but also in chronic corporal fibrosis in diabetes [23].

The overall lesson from all these models is the severe alteration of the cellular/ECM balance observed in the tunical fibroblasts and the corporal and arterial SMC, with myofibroblasts being clearly identified in the PD plaque [37,41,47], but

Tissue	Species	Condit	TGFβ	Ox Str	iNOS	Repl/apop
• Tunica	Human	PD	↗	↗	↗	
	Fisher rat	"	↗	↗	↗	
• Trabec	" "	Aging	--	↗	↗	↙
	ZDF fa/fa	"	--	↗	↗	↙
	Fischer rat	Diab 1	↗	↗	↗	↙
	iNOS ko	"			↙	↗
	Fischer rat	BCNR			↗	↙
• PDA	" "	Aging	↗	↗	↗	↙
	Zucker fa/fa	Diab II				↙

Figure 8 Fibrotic/antifibrotic balance in penile tissues.

apop = apoptosis; BCNR = bilateral cavernosal nerve resection; Condit = condition; Diab = diabetes; Ox Str = oxidative stress; iNOS = inducible nitric oxide synthase; ko = knockout mouse; PD = Peyronie's disease; PDA = penile dorsal artery; Repl = replication; TGFβ = transforming growth factor beta; Trabec = corporal trabecular tissue.

Tissue	Species	Cond	NO +	NO -	PDE5i
• Tunica	Fisher rat	PD	L-arg iNOS cDNA	L-NIL	varden silden
• Trabec	" "	Aging			silden
	ZDF fa/fa	Diab 2	Molsido	L-NIL	silden
	iNOS ko	"	Molsido	L-NIL	
	Fischer rat	BCNR		L-NIL	silden tadal varden
• PDA	" "	Aging		L-NIL	

BCNR = bilateral cavernosal nerve resection; cDNA = complementary DNA; Condit = condition; Diab = diabetes; iNOS = inducible nitric oxide synthase; ko = knockout mouse; NO+ = NO generator; NO- = iNOS inhibitor; PD = Peyronie's disease; PDA = penile dorsal artery; PDE5i = phosphodiesterase type 5 inhibitors; silden = sildenafil; tadal = tadalafil; Trabec = corporal trabecular tissue; varden = vardenafil.

Figure 9 Nitric oxide (NO)/cyclic guanosine monophosphate modulation of the fibrotic phenotype in penile tissues.

not as yet in the SMC tissues, as this may be obscured by the overall reduction in SMC. Similarly, the fibrotic process, indicated by TGFβ1 in some cases, oxidative stress and the proliferation/apoptotic ratio, is counteracted by the antifibrotic induction of iNOS and its products, NO and cGMP (see e.g., [34,41,51]). This is supported by the fact that blocking the iNOS endogenous mechanism of defense with L-NIL exacerbates fibrosis [9,10,38,39,42,52,53] (Figure 9). Also, inhibiting the action of members of the TGFβ superfamily, such as with shRNA for myostatin [49] or the TGFβ1 blocker decorin ameliorates penile fibrosis.

Other Novel Therapeutic Approaches to Prevent Fibrotic Processes in Penile Tissues

An emerging approach to treat corporal fibrosis is the replacement of the lost SMC by implanted stem cells (that can also be engineered ex vivo to express antifibrotic genes [59,60] (Figure 10). We recently showed that stem cells isolated from the skeletal muscle of mice can be implanted into the rat corpora cavernosa of old rats with ED and generate SMC [25]. By undergoing this conversion, the muscle-derived stem cells (MDSC) corrected the ED in the aged rats after even 4 weeks, as measured by electrical field stimulation of the cavernosal nerve. We are now studying the ex vivo gene engineering of MDSC with a series of genes directed by regulable promoters aimed to act via different antifibrotic mechanisms: elevation of

antifibrotic nitric oxide or cGMP levels (by iNOS cDNA or PDE5 shRNA) [61], or counteraction of the pro-fibrotic myostatin (by myostatin shRNA) [49,62,63]. The blockade of the Smad pathway, which is a common downstream signaling mechanism for both TGFβ1 or myostatin, is also a potential antifibrotic strategy, as upregulation of the expression of TGFβ1 and phospho-activation of the Smad pathway was shown to occur in the penis of the rat with streptozotocin-induced diabetes (a model for type 1 diabetes) [64]. Another promising approach is via the modulation of metalloproteinase expression by overexpression with the respective cDNA [65].

However, perhaps the most promising and novel approach is the pharmacological modulation of endogenous stem cells in the penis to produce SMC and to block myofibroblast generation. These cells have been identified in the rat penile tunica albuginea and trabecular tissue by immunohistochemistry and immunofluorescence for stem cell markers such as Sca1, and in

- ● Oxidative stress ➜ cytokine liberation ➜ chronic inflammation ➜ fibrosis ➜ loss of functional cells and tissue compliance
- ● Cell replacement by transplanted stem cells and/or pharmacological modulation of endogenous stem cells
- ● Ex vivo engineering for modulating stem cell lineage commitment

Figure 10 Stem cells in the therapy of tissue fibrosis and remodeling.

Tissue	Species	Cond	PPARγ agonist	TGFβ family	Oxid stress	Stem cells
• Tunica	Fisher rat	PD		Mst shRNA		
• Trabec	" "	Aging	Pioglit			MDSC
	ZDF fa/fa	Diab 2	Pioglit		Allopur	
	iNOS ko	"		Decorin	Allopur	

Figure 11 Other modulation of the fibrotic phenotype in penile tissues.

Allopur = allopurinol; Cond = condition; Diab = diabetes; iNOS = inducible nitric oxide synthase; Mst = myostatin; Oxid = oxidative; PD = Peyronie's disease; Pioglit = pioglitazone; PPARy = peroxisome proliferator activated receptor gamma; shRNA = small hairpin RNA; TGFB = transforming growth factor beta; Trabec = corporal trabecular tissue.

cell cultures from the human tunica [25,43]. The latter cultures have the ability in vitro to generate different cell lineages. We have identified by DNA microarrays another stem cell marker in the MDSC used for the previous experiments of stem cell implantation in the penis and also for vaginal regeneration [66]. This is Oct-4, an embryonic stem cell marker, used recently to program, together with three other embryonic stem cell genes, fibroblasts from adult skin into a multipotent stage similar to embryonic stem cells [67]. This has allowed us to identify putative stem cells in penile tissues that express Oct-4 and may be or may be not identical to the Sca1+, CD34+ cells previously detected by us [25,43], or the embryonic-like stem cells detected in a variety of adult tissues [68]. These endogenous stem cells may be good candidates for antifibrotic pharmacological modulation, particularly with agents belonging to the NO/cGMP and TGFβ1 pathway. We believe that this approach is not far-fetched and actually more feasible than regular gene and stem cell therapy for combating penile

- **Inhibit myofibroblast generation, replenish smooth muscle cells, counteract fibrotic phenotype, by:**
- **Nitric oxide generators**
- **Phosphodiesterase type 5 inhibitors**
- **Anti-transforming growth factor beta family/smad agents**
- **Cell differentiation master genes**

Figure 12 Pharmacological modulation of endogenous stem cell differentiation in the penis to counteract fibrosis.

fibrosis and restoring the normal cell compartments in both the corpora and the tunica.

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REVIEW

Rationale for phosphodiesterase 5 inhibitor use post-radical prostatectomy: experimental and clinical review

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Erectile dysfunction (ED) is a common complication after radical prostatectomy and results from trauma sustained by the cavernosal nerves. This is a major concern for patients and often affects treatment decisions. The likely mechanism for post-prostatectomy ED is through corporal veno-occlusive dysfunction. There is an increasing amount of evidence to suggest that phosphodiesterase 5 inhibitors (PDE5 inhibitors), when given on a continuous long-term basis, can help to prevent and reverse ED after surgery. In this review article we will examine the pathophysiology of post-prostatectomy ED and discuss the experimental and available clinical evidence for administering PDE5 inhibitors after prostatectomy.
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Erectile dysfunction is common following radical prostatectomy

Recent data suggest that approximately 161 000 men per year undergo a radical prostatectomy in the United States.[1] It is thought that many more men who are diagnosed with early-stage prostate cancer would accept this surgical form of treatment for their newly diagnosed disease if it were not for the possibility of the development of erectile dysfunction (ED). This common complication after radical prostatectomy is largely due to trauma sustained by the cavernosal nerves and is still widely encountered even after the most recent advances in surgical technique to spare the nerves.[2–5] Of men that are potent before surgery, only about 40–74% of men regain sexual function.[4–7] In fact, 41.9% of men reported that their sexual performance was a moderate to large problem after surgery[7] and patients appear to value sexual function so highly that they are often willing to choose therapy that offers better potency with lower life expectancy than

options that offer longer life expectancy and lower potency rates.[8]

Radical prostatectomy seems to disrupt and/or damage the neurovascular mechanisms responsible for eliciting an erection thereby resulting in either temporary or permanent ED postoperatively. In its mildest form, apparent in a successful bilateral nerve sparing prostatectomy, the trauma resulting from surgical manipulation of the neurovascular bundles may result in a neuropraxia leading to temporary ED. The most severe form of ED results from a non-nerve sparing prostatectomy where both nerves are transected, either volitionally or unrecognized at the time of surgery, and this leads to the complete loss of neuroregulatory functions in the corpora cavernosa.

CVOD is the most common form of ED following radical prostatectomy

Injury to the cavernosal nerves results in the atrophy and degradation of the underlying cavernosal smooth muscle, which, besides resulting in ED, may also lead to a decrease in penile weight.[9] Histologically, such a neuropraxia/neurotomy leads to apoptosis of the cavernosal smooth muscle and an excessive deposition of collagen within the cavernosa, which clinically results in corporal

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veno-occlusive dysfunction (CVOD). CVOD or venous leakage occurs because of the inability of the cavernosal smooth muscle cell mass to adequately compress the subtunical veins and prevent leakage of blood out of the cavernosa during tumescence. With CVOD, the patient complains of the inability to obtain and maintain an erection sufficient for completion of the sexual act and CVOD has been recognized as the major cause of ED subsequent to radical prostatectomy.[10,11]

Therefore, the poor response of many patients to the oral phosphodiesterase 5 inhibitors (PDE5) inhibitors given on demand post-radical prostatectomy could be due either to the neural injury which prevents the normal release of nitric oxide from the cavernosal nerve endings (a necessary requirement for the synthesis of the second messenger cyclic guanosine monophosphate (cGMP) within the cavernosa) or the subsequent loss of some of the corporal smooth muscle mass as elucidated above due to the neural injury or a combination of both conditions. In addition, the failure of vasoactive drugs injected intracorporeally into the penis to induce an erection in post-prostatectomy patients suggests that the corporal smooth muscle mass has most likely been impacted by the surgery in these patients. While arterial insufficiency post-prostatectomy due to intraoperative damage to the arteries supplying blood to the cavernosa is another means by which some of these men may experience ED,[12] it is CVOD that is the predominant cause of ED post-prostatectomy.

Although the hypoxia theory has been promulgated as the reason why there is a loss of corporal smooth muscle and an increase in collagen following this neural injury post-prostatectomy, the scientific evidence to show that intracellular hypoxia occurs during detumescence is very weak at best. This hypoxia theory states that low-oxygen tension occurs within the cavernosal tissue when there is detumescence and this leads to the induction of elevated levels of the profibrotic cytokine, transforming growth factor-β1 (TGFβ1), within the cavernosa.[13,14] However, to date, the only p02 that has been measured within the penis in such a setting is the sinusoidal p02 and there is no scientific evidence that the smooth muscle and other components of the cavernosal tissue obtain O_2 from the sinusoids rather than their own capillaries.

Antifibrotic role of NO following cavernosal nerve resection

The one theory that seems to be the most plausible in explaining why the corporal smooth muscle deteriorates in tandem with an increase in collagen content following radical prostatectomy is that it is the neural injury itself that induces proapoptotic

(loss of smooth muscle) and profibrotic (increase in collagen) factors within the cavernosa. It is possible that the ablation by neuropraxia of certain key growth factors produced by the cavernosal nerves may be responsible for eliciting the smooth muscle fibrosis and atrophy observed in corporal tissue. However, the production of cytokines and noxious agents by the damaged nerve axons may also be the causal factor of the increased early smooth muscle apoptosis,[15,16] which in turn may trigger collagen deposition to replace the lost cells. Similarly to the situation with skeletal muscle atrophy after denervation,[17,18] the molecular and cellular etiology of the tissue atrophy subsequent to cavernosal nerve damage remains to be elucidated.

In an attempt to counteract this proapoptotic and profibrotic cascade induced by such a neural injury, the cavernosal tissue itself then initiates an anti-apoptotic and an antifibrotic defense mechanism via the formation of nitric oxide and cGMP within the smooth muscle itself (Figure 1).[19] The key to erectile function post-radical prostatectomy in patients who are potent before the prostatectomy is maintenance of the integrity of the corporal histology (that is, prevention of both fibrosis and apoptosis of the smooth muscle). Therefore, in the post-prostatectomy patient, tumescence should be attainable au natural if both the nerves and blood vessels are not injured during the surgery. However, even if the nerves are injured but the histology of the cavernosal tissue can be preserved, as long as the arterial inflow is not impeded then tumescence in these patients may be achieved albeit with the use of intracorporeal injections and possibly with intraurethral applications of vasoactive substances.
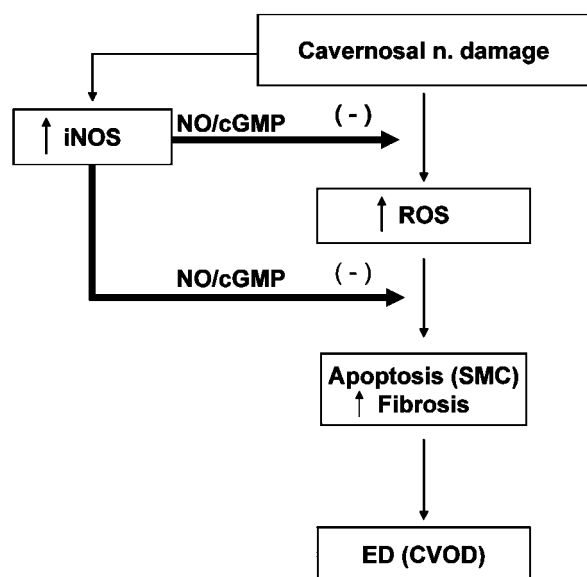

Figure 1 Effect of nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) from inducible isoform of nitric oxide synthase (iNOS) in combating reactive oxygen species (ROS), fibrosis and apoptosis. (−), inhibitory effect.

Nitric oxide, as the case of its subsequent downstream second messenger cGMP that also acts as an antifibrotic agent in the setting of cavernosal nerve injury, does not emanate from the nitrergic nerve endings of the cavernosal nerve but is induced by the smooth muscle itself.[18] The nitric oxide from the nerve endings of the cavernosal nerve is produced by the neuronal isoform of the nitric oxide synthase (nNOS) enzyme[20] whereas the nitric oxide that emanates from the cavernosal smooth muscle cells once the neural injury occurs is derived, at least in part, from the induction of the inducible isoform of nitric oxide synthase (iNOS).[15,16] There is a marked distinction between these two isoforms. While nitric oxide in the corpora during sexual stimulation is believed to be produced immediately upon sexual stimulation, albeit in small amounts, primarily by the activation of nNOS, the production of nitric oxide from iNOS in the corpora is very different from that of nNOS in that it is unrelated to sexual stimulation and occurs by transcriptional induction that results in the production of sustained amounts of nitric oxide, although somewhat delayed in its onset.

The evidence supporting the view that iNOS undergoes spontaneous induction in the corpora cavernosa in certain conditions such as aging, diabetes and specifically cavernosal nerve damage by protecting the histological and functional integrity of the corpora through combating fibrosis, stems from four main sources of experimental data. First, the fact that general inhibition of the activity of all NOS isoforms, and hence nitric oxide production, by prolonged sustained administration of N(G)-nitro-L-arginine methyl ester to rats, leads to considerable fibrotic degeneration in organs such as the heart, liver and kidney, independent of hemodynamic factors that may contribute to this process.[21–25] Second, specific genetic blockade of iNOS in the iNOS knockout mice leads to exacerbation of experimental fibrosis of the kidney and liver,[26,27] and the chronic inhibition of iNOS activity in rats by N6-(1-iminoethyl)-L-lysine dihydrochloride (L-NIL) intensifies aging-related fibrosis of the arterial wall and of the experimentally induced Peyronie's disease-like fibrotic plaque in the penis.[28,29] Third, administration of iNOS cDNA in the latter model reduces the fibrotic plaque.[30] Finally, specifically in cavernosal nerve damage, a similar treatment with L-NIL exacerbates CVOD and the underlying fibrosis of the corpora.[16]

It is assumed that prolonged endogenous induction of iNOS to moderate levels produces sufficient nitric oxide as to reduce collagen synthesis, quench reactive oxygen species (ROS), inhibit TGFβ1 expression and myofibroblast differentiation, and activate metalloproteinases that break down collagen.[17] If nitric oxide reaches excessive levels it may turn to be deleterious by causing cell death and oxidative stress, and this will depend on the tissue environment.

PDE5 inhibitors protect the integrity of the corporal smooth muscle following cavernosal nerve resection: experimental evidence

There is quite an amount of emerging scientific evidence to suggest that prolonged elevated levels of nitric oxide and cGMP can have an antifibrotic effect on a variety of tissues including tunica albuginea and corporal tissue (Figure 2).[19,20,31] Since PDE5 inhibitors work by inhibiting the enzyme that degrades cGMP, and since cGMP via activation of PKG inhibits collagen synthesis, this may be the preferential route of antifibrotic action when cGMP levels are maintained high for sustained periods. Although cGMP seems to stabilize iNOS mRNA or activate its transcription,[32] and thus may upregulate nitric oxide production, recent evidence suggest that this is not the case for the long-term effects of tadalafil on corporal fibrosis after cavernosal nerve damage in the rat.[16] Although there have been reports in the literature regarding the clinical effects of administration of these PDE5 inhibitors post-prostatectomy,[33–36] the rationale behind the use of these drugs on a prolonged and continuous basis in the post-prostatectomy patient has never been fully and scientifically delineated. The recent publication by Ferrini et al.[15] examined the effects of the administration of the PDE5 inhibitor, vardenafil, and demonstrated that the prolonged and continuous administration of the compound was effective in preventing both the fibrosis and loss of smooth muscle seen following bilateral cavernosal nerve resection (Figure 3). Compared with the sham group, the bilateral cavernosal nerve resection rats demonstrated a threefold increase in intracorporeal apoptosis, a 60% reduction in the smooth muscle to collagen ratio, a twofold increase in iNOS expression and development of CVOD (Table 1). When vardenafil was given daily for 45 days to the animals that underwent bilateral cavernosal nerve resection, CVOD did not develop and the abnormal corporal smooth muscle to collagen ratio seen in the bilateral cavernosal nerve-resected group was normalized. Similar results have been reported in the both the unilateral and bilateral nerve resection
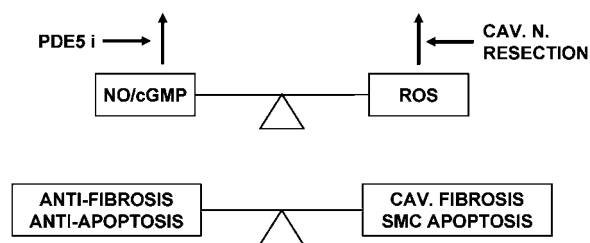

Figure 2 Relationship between reactive oxygen species (ROS) and nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) following cavernosal nerve resection.
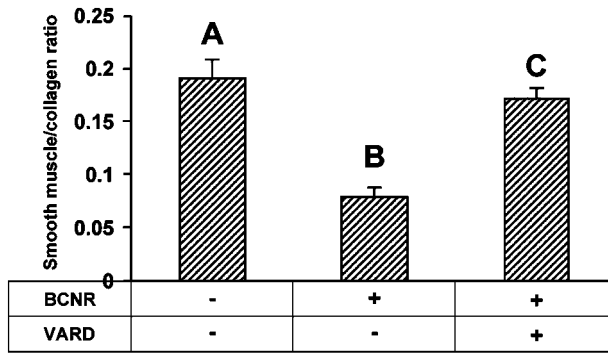
Figure 3 Effect of bilateral cavernosal nerve resection (BCNR) and vardenafil treatment on the smooth muscle/collagen ratio in the rat corpora cavernosa. Bilateral cavernosal nerve resection leads to a decrease in the smooth muscle/collagen ratio, which leads to the development of CVOD. The ratio becomes similar to the control group after treatment with vardenafil. Values determined by quantitative image analysis. BCNR (**b**) vs sham (**a**) or BCNR+V (**c**): $P < 0.001$; sham (**a**) vs BCNR+V (**c**): not significant. Ferrini et al.[15] with permission.

Table 1 Dynamic infusion cavernosometry

	ICP after papaverine (mm Hg + s.e.m.)	Drop rate (mm Hg/min)
Control ($n = 6$)	62 ± 8	18 ± 2
Bilateral nerve resection ($n = 7$)	64 ± 6	$42 \pm 5**$
Bilateral nerve resection + vardenafil ($n = 11$)	61 ± 2	$17 \pm 2*$

Abbreviation: ICP, intracavernosal pressure.
Dynamic infusion cavernosometrics in the rat after bilateral cavernosal nerve resection with and without continuous daily vardenafil for 45 days.
Drop rate was measured as the decrease in intracavernosal pressure in 1 min after cessation of saline infusion into the penis. This implies that the bilateral nerve-resected group has a large drop rate suggestive of a venous leak.
When treated with vardenafil, the drop rate is similar to the control group, thus suggesting that the CVOD has been normalized.
*Denotes $P < 0.05$ compared to BCNR and ** compared to sham operated. Ferrini et al.[15] with permission.

models using continuous long-term administration of sildenafil.[16]

A parallel study by Vignozzi et al.[37] also found that chronic tadalafil administration (120 days) to rats similarly reversed the decline in the cavernosal smooth muscle to collagen ratio that occurred after a bilateral cavernous neurotomy. Although Vignozzi et al. hypothesized that the effect of the tadalafil may have been due to the reversal of hypoxia induced by the neurotomy, these studies taken together suggest that PDE5 inhibitors can potentially protect and preserve the integrity of the corpora after cavernosal nerve damage when given on a prolonged and continuous basis.

PDE5 inhibitors protect the integrity of the corporal smooth muscle following cavernosal nerve resection: clinical evidence

The only clinical trial to suggest vaguely that chronic PDE5 inhibition post-prostatectomy may preserve the integrity of the corpora emanates from Schwartz et al.[35] who performed post-prostatectomy biopsies on men on chronic sildenafil treatment and found that the corporal smooth muscle to collagen ratio was maintained in those patients treated with chronic sildenafil while those who did not take sildenafil showed loss of smooth muscle content with a concomitant increase in collagenization of the corpora.

Clinical trials have also examined whether the routine use of PDE5 inhibitors on an on-demand basis to induce an erection post-prostatectomy, as opposed to its chronic use on a daily basis, may be beneficial long term in treating or correcting the ED that occurs after radical prostatectomy. Indeed, there are a number of such on-demand treatment studies using each one of the PDE5 inhibitors, vardenafil,[33] sildenafil[34] and tadalafil,[36] and each one touting the efficacy of their compound in improving post-prostatectomy potency rates. Although the theory promulgated by these latter nonrandomized, non-controlled studies to explain the efficacy of the PDE5 inhibitors is via cavernosal oxygenation, as enumerated earlier in this review there is as yet no direct scientific evidence to support that these compounds improve tissue oxygenation within the corporal tissue itself.

What is apparent from the emerging clinical and experimental data on the use of PDE5 inhibitors post-prostatectomy is that these drugs appear to play some role in preserving the integrity of the corporal tissue following cavernosal nerve damage (Figure 3). The importance of this observation is that regardless of whether the neural injury to the penis following surgery is permanent or not, preservation of the normal histology of the preoperative cavernosa will allow the corporal tissue to respond normally to the administration of locally administered proerectogenic agents even if the tissue should fail to respond normally to the on-demand agents. Emerging studies focusing on the molecular mechanisms of apoptosis and fibrosis are beginning to shed some light as to why the chronic and prolonged use of PDE5 inhibitors is proving to be beneficial. Obviously, further animal and randomized clinical studies are needed to confirm these exciting preliminary observations. A paradigm involving the discontinuation of the PDE5 inhibitor administration after the long-term post-prostatectomy treatment would allow one to verify whether indeed the improved erectile response is maintained in the total absence of the drug. The latter would suggest that the beneficial effects of this regimen with PDE5

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inhibitors on the underlying corporal histology seen in the rat model would also occur in men, and support the role of smooth muscle fibrosis in the etiology of CVOD.

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Sexual Medicine

Chronic daily tadalafil prevents the corporal fibrosis and veno–occlusive dysfunction that occurs after cavernosal nerve resection

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> Study Type – Aetiology (individual case
>   control study)
> Level of Evidence   3b

OBJECTIVES

To determine whether a long-term single daily oral dose of a longer half-life phosphodiesterase-5 (PDE5) inhibitor, tadalafil, has a similar effect to that of the shorter half-life PDE5 inhibitors sildenafil and vardenafil, and can prevent the fibrosis and resultant corporal veno-occlusive dysfunction (CVOD) occurring after cavernosal nerve (CN) injury.

MATERIALS AND METHODS

Male rats (10 per group) had either a sham operation, unilateral CN resection (CNR) or bilateral CNR, and were left untreated or given retrolingually 5 mg/kg per day of tadalafil. After 45 days, CVOD was assessed via cavernosometry, and the underlying corporal tissue changes were examined by immunohistochemistry and histochemistry

(followed by quantitative image analysis), Western blots, and ad hoc methods.

RESULTS

Tadalafil treatment normalized the low response to papaverine and high drop rate in the intracavernosal pressure measured by cavernosometry after CNR compared with sham-operated rats. Tadalafil also normalized the increase in penile shaft collagen content, and the reduction in corporal smooth muscle cell (SMC) content, SMC/collagen, and replication index, and improved the lower collagen III/I ratio and the increase in apoptotic index, caused by CNR, compared with sham operation. There were no effects of tadalafil on increased transforming growth factor β1, inducible nitric oxide synthase and xanthine oxidoreductase levels.

CONCLUSIONS

A long-term single daily dose of tadalafil prevented CVOD and the underlying corporal fibrosis in the rat caused by CN damage, as

effectively as the previously reported continuous treatment with vardenafil or sildenafil, through a cGMP-related mechanism that appears to be independent of inducible nitric oxide synthase induction.

KEYWORDS

erectile dysfunction, nerve-sparing radical prostatectomy, PDE5 inhibitors, inducible nitric oxide synthase, fibrosis, smooth muscle

INTRODUCTION

Radical prostatectomy (RP) is considered by many to be curative for patients with early-stage prostate cancer. However, because of the potential risk of damage to the cavernosal nerves (CNs) and the subsequent development of erectile dysfunction that affects 60–90% of patients at 1 year after RP [1–4], this surgical option is often declined. In a large study, there was recovery of potency after 5 years in only 28% of cases [2]. Although nerve-sparing (NS) techniques have been developed that attempt to reduce the incidence of erectile dysfunction after RP, its success is not guaranteed and depends on the surgical centre, age of patients, and other factors, being 31–86% of cases in bilateral NS retropubic RP (NSRRP), to 13–56% in unilateral NSRRP [1,3].

Most of the potency rates cited above correspond to patients treated with oral phosphodiesterase-5 (PDE5) inhibitors given on demand to elicit an erection, with 35–75% response rates for sildenafil given after NSRRP, vs 0–15% for non-NSRRP [5,6]. Other reports give responses of 40–50% with vardenafil or tadalafil, after bilateral NSRRP, although these have not been direct comparisons, and patient selection criteria varied [7]. Most of these men, and those who do not respond to the PDE5 inhibitors, have vasculogenic erectile dysfunction when evaluated by duplex ultrasonography and/or dynamic infusion cavernosometry. In one such study, 59% of men showed arterial insufficiency after bilateral NSRRP and a substantial fraction (26%) had 'venous leakage' or corporal veno-occlusive dysfunction (CVOD) [8]. The latter group had the worst prognosis for the return of erectile function 1 year after RP (only 9%).

The clinical evidence suggests that in addition to the CN damage that can occur as a result of RP, the surgically elicited neuropraxia can also lead to alterations within the corpora cavernosa, specifically loss of smooth muscle (SM) and excessive collagen deposition, as well as a putative endothelial damage to the sinusoids [9–11]. Indeed, experimental studies conducted in the rat showed that CN damage elicited by either resection, freezing or crushing, is accompanied by a complete or partial reduction in the erectile response to electrical field stimulation of the CN and by profound histological changes within the corpora [12–16], similar to that in the human corpora after RP. These alterations consist of a loss of corporal SM cells (SMC) by apoptosis, and an increase in collagen deposition within the corpora, e.g. tissue fibrosis, and this is presumed to be the cause of CVOD in patients after RP.

We have shown in the rat that CN resection (CNR) leads to a spontaneous induction of the inducible nitric oxide synthase (iNOS, also known as NOS2) within the cavernosal SMC [15,16], and proposed that it acts as an antifibrotic compound that attempts to protect the corpora cavernosal histology in the same way that it acts on other tissues undergoing fibrosis, e.g. the penile tunica albuginea, the vagina, or the peripheral arteries [17–22]. iNOS expression presumably produces a steady increase in local NO, that also raises local cGMP levels, and these two products inhibit collagen production and preserve the SM. In the case of NO, it quenches the pro-fibrotic reactive oxygen species generated during oxidative stress.

In this CNR rat model, when the short-acting PDE5 inhibitors, sildenafil or vardenafil, were used long-term and continuously, rather than 'on-demand' as used clinically to induce an erection in men, the histology of the corporal tissue and the dynamic infusion cavernosometric responses in these rats became normal, presumably by increasing local cGMP levels [15,16]. This agrees with and might explain the results obtained by Padma-Nathan [7], giving nightly sildenafil for 9 months to patients with bilateral NSRRP, where after a 4-week discontinuation of treatment, 27% of patients had a return of spontaneous erections.

In the present study, we aimed to determine whether the long-acting PDE5 inhibitor, tadalafil [23,24], given in daily single doses

orally, rather than continuously as in the previous studies [15,16], was also effective in preserving the integrity of the corporal histology and the erectile response of rats treated with unilateral (U) or bilateral (B)CNR.

MATERIALS AND METHODS

Fisher 344 male rats (5 months old; Harlan Sprague-Dawley, San Diego, CA, USA) were treated with an institutionally approved protocol, and randomly divided into the following five groups (10/group): A (sham-operated); B (UCNR), C (UCNR + tadalafil), D (BCNR), and E (BCNR + tadalafil); the CNR was done as described previously [15,16]. In group A, both CNs were identified but not resected. In groups D and E, both CNs and ancillary branches were resected by removing a 3-mm segment, whereas in the respective UCNR groups (B and C) only one of these nerves was resected. Tadalafil (Lilly ICOS, San Francisco, CA, USA), was dissolved in 10% glucose/1% Tween 80 and administered retrolingually once per day, as described previously for vardenafil in another model [25]. The daily tadalafil dose given to these rats (5 mg/kg/day) was about equivalent to a single 50 mg daily dose in men, when corrected for differences in total body surface area [15,16,26]. Treated rats had their tadalafil suspended 3 days before cavernosometry and death, as a 'washout'.

As previously described [15,17,27], the basal intracavernosal pressure (ICP) was recorded, and 2 mg papaverine was administered through a cannula into the corpora cavernosa. The ICP was recorded 5 min later as the 'ICP after papaverine'. After complete detumescence, saline was then infused through another cannula, increasing the infusion rate by 0.05 mL/min every 10 s, until the ICP reached 100 mmHg ('infusion rate'). Then the infusion was adjusted to hold the ICP at ≈100 mmHg ('maintenance rate'). The rate of decrease ('drop rate') was determined by recording the decrease in ICP within the next 1 min after the infusion was stopped.

After cavernosometry, the rats were killed and the middle regions of the skin-denuded penile shafts were fixed overnight in 10% formalin, washed, and stored in 70% alcohol at 4 °C until processed for paraffin-embedded tissue sectioning (5 μm). Adjacent sections were used for Masson's trichrome staining for

FIG. 1. *Effect of long-term treatment with a single daily dose of tadalafil on the erectile function of the rat measured by pharmacological and dynamic infusion cavernosometry. Tadalafil treatment was given daily for 45 days, with a 3-day previous washout. Top, response of the ICP to papaverine. Bottom, response of the ICP to the interruption of saline infusion. Groups are as defined in the Methods. P *< 0.05; **< 0.01; ***< 0.001.*
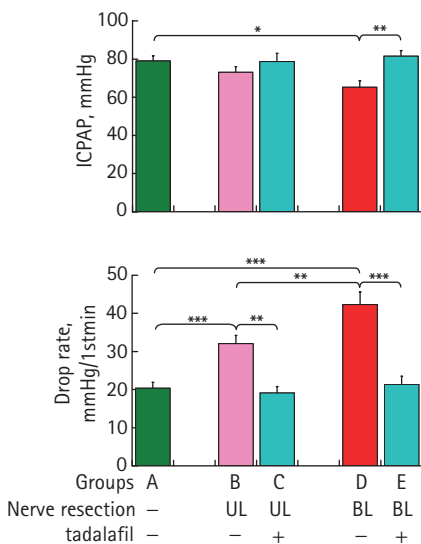
collagen (blue) and smooth muscle (red); picro-sirius red under polarized microscopy for collagen III (green and green-yellow) and I (red and orange) ratios; and immunodetection with monoclonal antibodies against α-SM actin (ASMA) as a SMC marker (Sigma Diagnostics, St. Louis, MO, USA), proliferating cell nuclear antigen (PCNA) as a marker of cell proliferation (Chemicon, Temecula, CA, USA), and polyclonal antibodies against TGFβ1 (Promega, Madison, WI, USA), and iNOS (Calbiochem, La Jolla, CA, USA), and the oxidative stress marker xanthine oxidoreductase [15–21,28]. The specificity of the antibodies was validated by Western blot.

Sections were then incubated with biotinylated antimouse IgG for ASMA and PCNA or biotinylated antirabbit IgG for iNOS and TGFβ1, followed by avidin-biotin complex (Vector Laboratories, Temecula, CA, USA) and 3,3'diaminobenzidine (Sigma) for PCNA and iNOS, or the ASMA Sigma Kit for ASMA and 3-amino-9-ethylcarbazole. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) assay was used with the Apoptag peroxidase detection assay (Chemicon) [15–21]. The sections were counterstained

with haematoxylin. For the negative controls for immunohistochemistry the first antibody was replaced with the IgG isotype. For the negative control for the TUNEL assay, buffer was substituted for the terminal deoxynucleotidyl transferase enzyme. Testicular sections from old rats were used as a positive control for TUNEL.

For quantitative image analysis we used computerized densitometry (ImagePro Plus, version 5.1, Media Cybernetics, Silver Spring, MD, USA) coupled to a microscope equipped with a digital camera [15–21]. For Masson staining, ×100 views of the penis, composed of one half of the corpora cavernosa but excluding the sinusoidal spaces, were analysed for SM (stained red) and collagen (stained blue) and expressed as the SM/collagen ratio. An identical approach was used for the collagen III/I ratios. For ASMA, xanthine oxidoreductase and iNOS staining, only the corpora cavernosa was analysed in a computerized grid, and expressed as the percentage of positive area vs the total area of the corpora cavernosa. The intensity of immunostaining was determined as the percentage of integrated optical density in the corpora cavernosa. For the TGFβ1, PCNA and TUNEL determinations, the number of positive cells at ×200 was counted, and the results expressed as the percentage of positive cells/total cells in the corpora cavernosa. In all cases, four fields at ×100, or eight fields at ×200, were analysed per tissue section, with at least four matched sections per rat and 6–10 rats per group.

Penile homogenates of frozen tissue (100 mg) were obtained in a 1% SDS buffer and protease inhibitors (3 μM leupeptin, 1 μM pepstatin A, 1 mM phenyl methyl sulphonyl fluoride), and centrifuged at 10 000 g for 5 min [16–21,26]. Supernatant protein (30 μg) was run on 7.5% or 10% (ASMA) polyacrylamide gels, and submitted to Western blot immunodetection with a monoclonal ASMA IgG (1 : 1000; Oncogene-Calbiochem), detecting a 43-kDa band. Membranes were incubated with a secondary polyclonal horse antimouse IgG linked to horseradish peroxidase (1 : 2000; BD Transduction Laboratories), and bands were visualized with luminol (Pierce, Rockford, IL, USA) [16–21,26]. A single positive control was run throughout all gels for each antibody to standardise for variations in exposures and staining intensities. For negative controls

the primary antibody was omitted. Band intensities were determined by densitometry and corrected by the respective intensities for a housekeeping protein, glyceraldehyde phosphate dehydrogenase (GAPDH), upon reprobing.

For collagen estimation in fresh tissue, as previously described, the tissue was homogenized in saline, hydrolysed with 2 M NaOH for 30 min at 120 °C, followed by the estimation of hydroxyproline by a modification of the Neumann and Logan's reaction using Chloramine T and Ehrlich's reagent, against a hydroxyproline standard curve and measuring at 550 nm [16,21,26]. Values were expressed as μg of collagen per mg of tissue.

The values are expressed as the mean (SEM); the normality distribution of the data was established using the Wilks-Shapiro test. Multiple comparisons were analysed by a single-factor ANOVA, followed by post hoc comparisons with the Newman-Keuls test, with differences considered significant at $P < 0.05$.

RESULTS

CNR lead to moderate CVOD and underlying corporal fibrosis that was prevented by long-term daily treatment with tadalafil. No side-effects, e.g. lethargy, priapism, aggressiveness, or hyperactivity were noted in the rats treated with tadalafil. Compared with group A, the peak ICP after papaverine administration was significantly reduced by BCNR (Fig. 1, top D vs A), but remained virtually unchanged by UCNR (top B vs A). Tadalafil administered beginning on day 1 after surgery restored, in the BCNR rats, the normal ICP seen in group A (E vs A). On saline infusion into the detumescent penis, the drop rates were very low in group A (bottom, Fig. 1A), confirming normal CVO. However, in the UCNR rats, the drop rate was 1.5 times higher than in group A (B vs A), suggesting a moderate CVOD, and this was increased to more than twice the difference after BCNR (D vs A). In both UCNR and BCNR, tadalafil restored the normal drop rates (C and E vs A).

There was a lower relative area occupied by SMC vs collagen (SMC/collagen ratio) in the corpora cavernosa on with Masson trichrome staining in the BCNR group than in group A (Fig. 2 top, D vs A), and this reduction was

FIG. 2. *Effect of long-term treatment with a single daily dose of tadalafil on the SM/collagen ratio and SMC content in the rat corpora cavernosa. Top panels: Tissue sections from the groups as shown in Fig. 1, were stained with Masson's trichrome: collagen blue, SMC red (arrows; ×100, bar = 200 μm). Bottom panels: Other adjacent sections were immunostained for ASMA as a SMC marker (arrows; ×200, bar = 100 μm). The micrographs depict representative fields and the bar plots show the quantitative image analysis. P, *< 0.05; **< 0.01; ***< 0.001.*

FIG. 3. *Effect of long-term treatment with a single daily dose of tadalafil on the expression of ASMA in total penile shaft tissue from rats after BCNR. Homogenates from total penile shaft tissue were assessed by Western blot for ASMA. Top: representative pictures of the gels depicting the ASMA band, and the housekeeping GAPDH band. Bottom: densitometry analysis. **P < 0.01.*

apparently counteracted by tadalafil (E vs A). In both the UCNR and BCNR groups, image analysis showed that the decrease in SMC/collagen was moderate (35–40%) (bottom, B and D vs A), but tadalafil (C and E) virtually normalized this ratio. The changes in the SMC/collagen ratio induced by BCNR were due mostly to a considerable decrease in the SMC compartment, as shown by immunodetection for ASMA in adjacent tissue sections, which was partly prevented by tadalafil (Fig. 3 top D and E, vs A). Image analysis confirmed the visual inspection, with an ≈70% decrease in ASMA staining in both UCNR and BCNR, a change that was reduced to only 39% in both groups on tadalafil treatment (Fig. 3, middle). To corroborate the effect of tadalafil on the SMC, the expression of ASMA was estimated by Western blot in total penile shaft homogenates that contained SM not only from the corpora cavernosa but also from the corpus spongiosum and the media of the penile arteries. In the BCNR rats, Fig. 3 (bottom) shows a representative view of the 42-kDa

band for ASMA in some of the specimens from the tadalafil-treated (E) vs untreated (D) rats, indicating that tadalafil increased the ASMA content by 60%.

The total content of collagen was evaluated in penile shaft tissue hydrolysates by the hydroxyproline assay (Fig. 4). UCNR (B) and BCNR (D) increased collagen by 19% and 59%, respectively, compared with group A, and tadalafil reduced these values even below (C and E) the normal content. However, there were no significant changes in the collagen III/I ratio with CNR or tadalafil treatment, as evaluated by the picro-sirius red staining/ polarized light visualization of the corpora, where collagen III is seen in green/greenish yellow and collagen I in orange/yellowish (not shown).

The loss of corporal SM induced by CNR was associated with an increase in apoptosis and a reduction in cell replication, and this was counteracted by long-term daily tadalafil treatment.
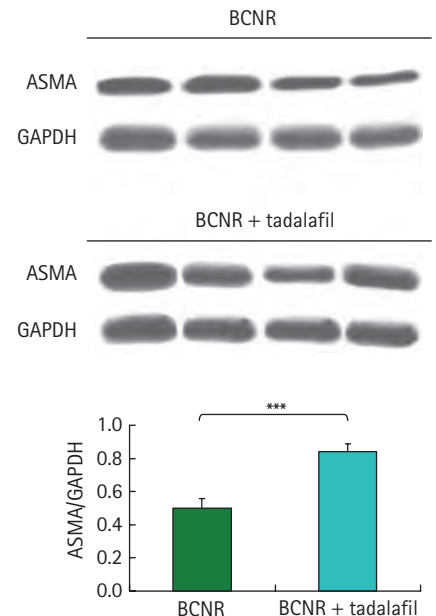
The effects of tadalafil on corporal SMC apoptosis were assessed with the TUNEL assay, as depicted in Fig. 5, which shows greater programmed cell death after BCNR than in group A, and this in turn was reduced by tadalafil (top, E and D, vs A). Image analysis showed a 75% and 95% increase in the apoptotic index by UCNR and BCNR, respectively (bottom, B and D vs A), and treatment with tadalafil reduced these values to a level not significantly different from those in group A. PCNA immunostaining was used to determine corporal cell proliferation, indicating considerably less after BCNR than in group A, which was counteracted by daily tadalafil treatment (Fig. 6 top micrographs, E and D, vs A). UCNR and BCNR led to a 73% and 70% reduction in the replication index compared with group A (middle, B and D, vs A), and tadalafil (E and C) increased those values above those in group A. As a result of these changes, the ratio between the replication and apoptotic indexes was reduced by >80% by both UCNR and BCNR (bottom, B and D vs A),

FIG. 4. Effect of long-term treatment with a single daily dose of tadalafil on the total collagen content and the collagen III/I ratio in the rat corpora cavernosa. Frozen penile specimens were used to determine the total collagen content by a hydroxyproline assay (top bar graph). Adjacent tissue sections to those in Fig. 2 were stained with picro-sirius red and visualized under polarized light (bottom bar graph). The bar plots show the quantitative image analysis. Groups and P values as in Fig. 1.

FIG. 5. Effect of long-term treatment with a single daily dose of tadalafil on apoptosis and TGFβ1 expression in rat corpora cavernosa. Adjacent tissue sections to those in the preceding figures were immunostained for TGFβ1, and other sections assessed by TUNEL staining. The bar plots show the quantitative image analysis. Groups and P values as in Fig. 1.
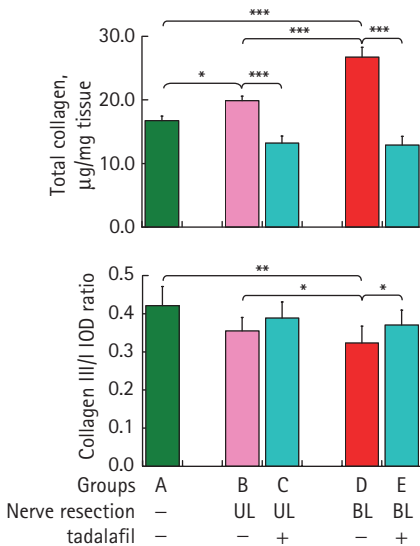
and tadalafil partly counteracted this reduction (C and E).

As we did not use dual immunohistochemistry for ASMA and TUNEL or PCNA, it is not possible to determine whether the changes in cell apoptosis and proliferation occurred in the SMC, but the location of this staining around the cisternae suggests that this was mainly the case, as SMC are the main cellular component in the corpora cavernosa.

Fibrosis of the corpora cavernosa induced by CNR was accompanied by an increased expression of some fibrotic and antifibrotic factors which are not affected by long-term daily treatment with tadalafil. BCNR led, as expected, to a considerable increase in the expression of one of the main pro-fibrotic factors, TGFβ1, but tadalafil treatment did not reduce this level, as shown in representative views (Fig. 7 top micrographs, D and E vs A). Image analysis indicated that UCNR and BCNR led to a similar increase in TGFβ1 expression, of 75–90%, as in group A

(B and D vs A), but tadalafil had little effect on these values (C and E). Similarly, xanthine oxidoreductase, a marker for oxidative stress in corporal tissue, was only moderately but not significantly increased by BCNR, and was not significantly changed by tadalafil, as evaluated by quantitative immunohistochemistry (not shown).
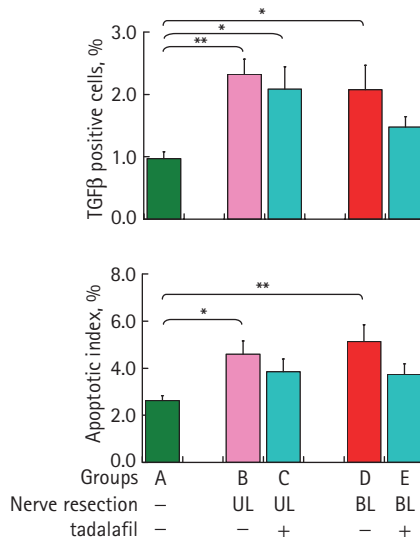
The increase in TGFβ1 by BCNR was paralleled by a very considerable induction of iNOS, a putative antifibrotic factor, and this process was not reduced by tadalafil (Fig. 7 bottom micrographs, D and E vs A). BCNR and UCNR actually increased iNOS by 3–4 times (D and B vs E), but tadalafil had no effect on the expression of iNOS (E and C).

DISCUSSION

The present results support and extend our previous results in rats subjected to cavernosal nerve damage but treated long-term and continuously with the short-acting PDE5 inhibitors, vardenafil [15] and sildenafil [16], given in the drinking water, showing that long-term treatment with a single daily dose

of the long-acting PDE5 inhibitor, tadalafil, induces a similar effect, i.e. the CVOD and underlying histological changes induced by the neuropraxia can be prevented. Our functional results agree with the findings in men after NSRRP treated with long-term daily sildenafil [7] and with the recovery of SM content by a similar treatment [10], and suggest how preventing corporal fibrosis might underlie the recovery of erectile function reported in the human study.

Our results from this rat model suggest that prolonged treatment with single frequent doses of tadalafil might be considered in the clinical setting to preserve the detrimental effects of RP on corporal tissue. Whether tadalafil is given daily, as in the current study, or more sporadically, based on the 3 day-efficacy of tadalafil due to its long half-life [23,24], needs to be determined. The latter might be more practical than for the other PDE5 inhibitors, although sildenafil taken every other night has been shown, as noted, to preserve corporal histology in men after RP [10]. Although the daily doses of vardenafil, sildenafil and tadalafil given in these rat experiments were 2–2.5 times higher (when corrected by surface area) than the usual or accepted daily dosage normally given to men for the on-demand treatment of erectile dysfunction [23,24], no side-effects of the drugs were seen in any of these studies.

The experimental BCNR rat model selected for this and our previous studies using vardenafil and sildenafil represents an extreme condition of nerve injury in which the CNs are resected rather than simply damaged and left *in situ* [12–16]. We chose this method of injury to assure reproducibility among rats, as it is less likely to vary among different laboratories than would any of the other models of crush injury described previously. In addition, any favourable response to PDE5 inhibitor treatment in the BCNR model suggests that the beneficial effects might even be enhanced, or require lower doses, in a less severe nerve injury that might occur experimentally with the crush models or even in the clinical setting. However, this would also have to be validated to answer at least three questions: (a) is treatment that is initiated after the consequences of the RP-induced neuropraxia on erectile function and corporal smooth muscle become evident, effective in correcting rather than preventing those changes; (b) for how long can treatment be interrupted once normal

FIG. 6. *Effect of long-term treatment with a single daily dose of tadalafil on cell proliferation and turnover in the rat corpora cavernosa. Top panels: adjacent tissue sections to those in the preceding figures were submitted to PCNA immunostaining (×200, bar = 100 μm, the arrows indicate PCNA-positive cells), and the ratio between the proliferation index and the apoptotic index calculated from Fig. 5 was plotted (bottom bar plots). The micrographs depict representative fields and the bar plots show the quantitative image analysis. Groups and P values as in Fig. 1.*

FIG. 7. *Effect of long-term treatment with a single daily dose of tadalafil on iNOS expression in the rat corpora cavernosa. Adjacent tissue sections to those in the preceding figures were immunostained for iNOS. The micrographs show representative fields (×40, bar = 500 μm, the arrows indicate iNOS-positive areas) and the bar plots show the quantitative image analysis. Groups and P values as in Fig. 1.*
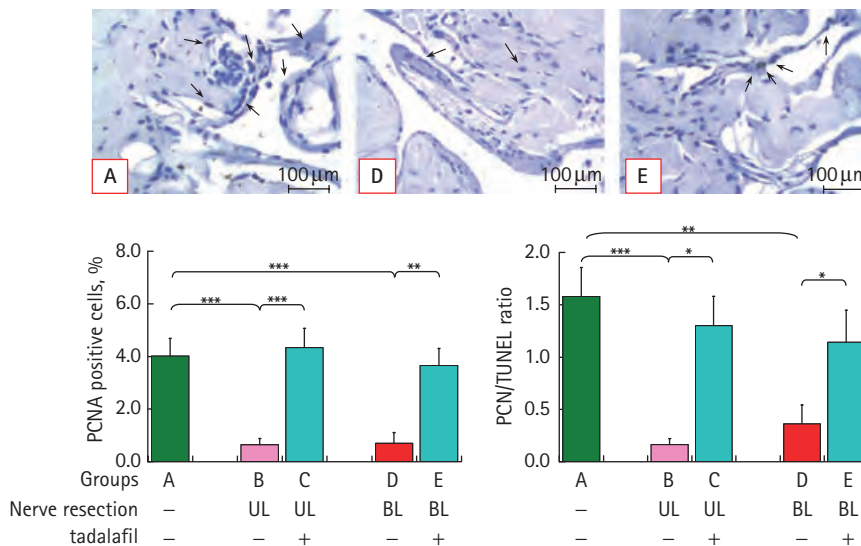
function is achieved without the risk of a relapse; and (c) are these effects limited to corporal fibrosis, or might the PDE5 inhibitors also facilitate CN regeneration by an alternative mechanism?.

Interestingly, the results obtained previously with vardenafil and sildenafil [15,16], and now tadalafil, given under two different regimens, are qualitatively equivalent in terms of preventing CVOD and the underlying histological alterations. The values of the 'drop rates' determined by cavernosometry in BCNR rats subjected to treatment were identical and in the normal range, as in group A, thus indicating that the CVOD induced by CNR was corrected to the same extent, albeit that vardenafil was slightly less effective in restoring a normal response to intracavernosal papaverine.

The findings were similar for the normalization of the collagen III to I ratio, and for the lack of a significant effect on iNOS expression. However, the single daily dose of tadalafil was more effective than sildenafil in reducing collagen deposition (the effects of vardenafil on collagen deposition were not measured) and stimulating cell proliferation, but was less effective in normalizing ASMA expression or reducing apoptosis. The overall

cell turnover measured by the proliferation/apoptosis ratio was protected to the same extent by the three drugs. Finally, tadalafil in this regimen did not reduce the TGFβ1 level, whereas sildenafil did (the assay was not used in the vardenafil study). TGFβ1 does not seem to be in the BCNR/UCNR corpora cavernosa as a critical pro-fibrotic factor, as seen in animal models of diabetes [29,30], or in the tunica albuginea in Peyronie's disease [17,18,20,31,32], or even in the vagina of the diabetic rat [21,33].

As to the mechanism of the effect of long-term PDE5 inhibitors in protecting the corporal cellular/extracellular balance and hence compliance of the corpora, it is most likely due to cGMP stimulating SMC replacement and reducing collagen synthesis via phosphokinase G activation [34–37], rather than acting via iNOS induction. Our previous studies showed that iNOS is used as an endogenous cellular defence to counteract fibrosis, as L-NIL (an inhibitor of iNOS activity) increases CVOD and corporal fibrosis in BCNR [16]. Further proof of this antifibrotic effect of iNOS is seen in the iNOS knockout mouse, where there is an increase in fibrosis in many organs, including the kidney and liver, and even in the aged penis [38–40]. This antifibrotic effect of iNOS would occur by the
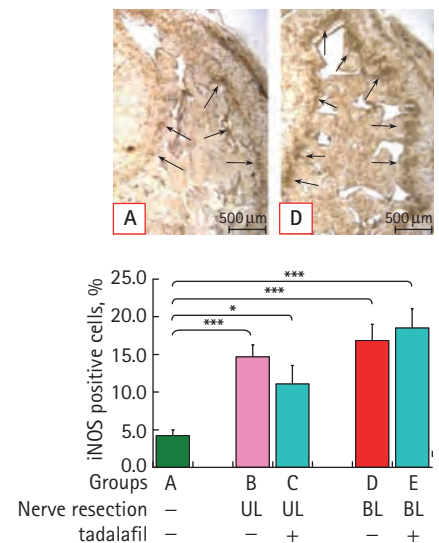
two processes mentioned for cGMP, either directly through NO release or indirectly through cGMP synthesis, plus the reduction of profibrotic reactive oxygen species in oxidative stress.

Despite the evidence cited above, the relative lack of effect of long-term PDE5 inhibitors on iNOS induction in the corpora of the BCNR rats, as reported here and in a previous study [16], and particularly the previous evidence that L-NIL does not significantly reduce the protective effects of long-term sildenafil on erectile function and the SMC/collagen ratio, make it unlikely that the protective effects of PDE5 inhibitors on the corpora would involve iNOS induction, at least in the BCNR rat model. This would differ from other conditions, like diabetes [29], where oxidative stress in the corpora is more significant than in BCNR. Therefore, in CN damage, the pharmacological increase of cGMP levels by PDE5 inhibitors and the endogenous iNOS induction might act in tandem. However, the latter process does not seem to be essential for maintaining sufficient cGMP to counteract fibrosis in the presence of PDE5 inhibition, as cGMP can be formed from many sources other than via NO from iNOS.

In conclusion, long-term treatment with a single daily dose of tadalafil appears to be as effective as the continuous treatment with vardenafil or sildenafil in preventing CVOD and corporal fibrosis after CN damage, and has more direct clinical relevance. Further studies are needed to decide whether this is related to the long pharmacological half-life of tadalafil, or if the same effects can be achieved with a similar regimen with vardenafil or sildenafil.

CONFLICT OF INTEREST

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Abbreviations: **ASMA**, α-smooth muscle actin; **(B)(U)CN(R)**, (bilateral) (unilateral) cavernosal nerve (resection); **CVOD**, corporal veno-occlusive dysfunction; **GAPDH**, glyceraldehyde phosphate dehydrogenase; **ICP**, intracavernosal pressure; **(i)NO(S)**, (inducible) nitric oxide (synthase); **PCNA**, proliferating cell nuclear antigen; **PDE5**, phosphodiesterase 5; **SM(C)**, smooth muscle (cells); **TUNEL**, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling; **(NS)(R)RP**, (nerve-sparing) (retropubic) radical prostatectomy.

ORIGINAL ARTICLE

Long-term continuous sildenafil treatment ameliorates corporal veno-occlusive dysfunction (CVOD) induced by cavernosal nerve resection in rats

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It was recently reported in the rat that vardenafil given in a continuous long-term manner was successful in preventing smooth muscle fibrosis in the penile corpora cavernosa and corporal veno-occlusive dysfunction (CVOD) that occur following bilateral cavernosal nerve resection (BCNR), a model for human erectile dysfunction after radical prostatectomy. To expand on this finding and to determine whether this effect was common to other PDE5 inhibitors, and occurred in part by stimulation of the spontaneous induction of inducible nitric oxide synthase (iNOS, also known as NOS2), male Fischer 344 rats ($N = 10$/group) were subjected to either BCNR or unilateral cavernosal nerve resection (UCNR) and treated with sildenafil ($20\,\mathrm{mg\,kg^{-1}\,day^{-1}}$) in the drinking water daily for 45 days. Additional BCNR groups received L-NIL ($6.7\,\mathrm{mg\,kg^{-1}\,day^{-1}}$) as inhibitor of iNOS activity, with or without concurrent sildenafil administration. It was determined that sildenafil, like vardenafil, (1) prevented the 30% decrease in the smooth muscle cell/collagen ratio, and the 3–4-fold increase in apoptosis and reduction in cell proliferation, and partially counteracted the increase in collagen, seen with both UCNR and BCNR; and (2) normalized the CVOD, measured by dynamic infusion cavernosometry, induced by both BCNR and UCNR. The long-term inhibition of iNOS activity exacerbated corporal fibrosis and CVOD in the BCNR rats, but sildenafil functional effects were not affected by L-NIL. These data suggest that the salutary effects of continuous long-term PDE5 inhibitors on erectile function post-cavernosal nerve resection involve their ability to prevent the alterations in corporal histology induced by cavernosal nerve damage, in a process apparently independent from endogenous iNOS induction.
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Introduction

Erectile dysfunction is a common complication following radical prostatectomy that affects the quality of life of both the patient and his partner. In addition, many men who have been diagnosed with early stage prostate cancer and are candidates for radical prostatectomy avoid this surgery primarily because of the fear of developing this side effect.[1–3] The main cause of erectile dysfunction in this patient population is corporal veno-occlusive dysfunction (CVOD), which occurs when the corporal smooth muscle is unable to relax sufficiently and let the intracorporeal pressure adequately compress the subtunical veins, which prevents the egress of blood out of the corpora during tumescence.[4] Regardless of its cause, when the number of smooth muscle cells (SMC) decreases and/or the collagen content increases, the corporal tissue loses its normal compliance and is prone to developing CVOD.

During a radical prostatectomy, the cavernosal nerves are susceptible to injury. This not only impairs

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the normal nitrergic neurotransmission which initiates the normal erectile response, but can also lead to the loss of SMC and corporal fibrosis.[5–8] It is this alteration in the corporal smooth muscle to collagen ratio that is assumed to lead to CVOD.[9] As a result, both spontaneous erections and the response to vasoactive drugs, including the oral PDE5 inhibitors when given on demand to elicit an erection, can be adversely affected.[3,10]

Several recent studies addressing CVOD in the aged or diabetic rat suggests that this form of erectile dysfunction is associated with the loss of SMC and excessive deposition of collagen fibers within the corpora, and that the long-term continuous administration of PDE5 inhibitors may counteract these processes.[11–13] It is likely that this occurs through the maintenance of high levels of cGMP, since this compound reduces collagen synthesis and the activation of the pro-fibrotic TGFβ1 pathway and protects SMC from apoptosis, while stimulating the spontaneous induction of inducible nitric oxide synthase (iNOS, also known as NOS2).[14–23] The expression of iNOS in certain non-immunological tissues is assumed to be a defense mechanism against fibrosis.[24–29] The nitric oxide produced by iNOS, besides inhibiting collagen synthesis and the TGFβ1 pathway, also quenches reactive oxygen species, and in some cases, the differentiation of fibroblasts to myofibroblasts, the cells that produce collagen in many fibrotic conditions.[30–32]

In a recent study, we have shown that the PDE5 inhibitor, vardenafil, given for 45 days in the drinking water to rats subjected to bilateral cavernosal nerve resection (BCNR)[33–35] prevented the development of CVOD and the underlying SMC loss and fibrosis in the corpora cavernosa.[36] An antifibrotic effect by vardenafil and sildenafil was observed in the penile tunica albuginea in the rat model of Peyronie's disease, and in the case of sildenafil in the aged corpora smooth muscle.[37,38,12] In order to confirm and expand those findings by studying not only BCNR, but also unilateral cavernosal nerve resection (UCNR), and by using another PDE5 inhibitor, we have determined: (a) how a unilateral nerve injury compares to the more severe BCNR, and (b) whether sildenafil has similar anti-fibrotic properties as vardenafil and works via iNOS induction.

Materials and methods

Animal treatments
Five-month-old male Fisher 344 rats (Harlan Sprague–Dawley, San Diego, CA, USA) were treated with an IACUC-approved protocol, and divided as follows ($n = 10$/group): A (sham-operated); B (UCNR), C (UCNR + sildenafil), D (BCNR) and E (BCNR + sildenafil). The drug was given in the drinking water for 45 days (water intake ad libitum). Nerve resection was

performed as described.[33,35,36] In the sham-operated group, both cavernosal nerves were identified but not resected. In the other groups, the main cavernosal nerves were resected by removing a 3-mm segment uni- or bilaterally. Sildenafil (Pfizer Ltd, Sandwich, UK) was dissolved in the drinking water ($0.3 \, mg \, ml^{-1}$), as described previously.[12] The drinking volume was determined daily, and the body weight was recorded weekly. The daily sildenafil dose ($20 \, mg \, kg^{-1}$) was approximately equivalent to a single 200-mg tablet daily dose in men, when corrected for differences in total body surface area.[12,38] Treated animals were switched to regular drinking water 1 day prior to cavernosometry, as a washout process. In a subsequent experiment, treatments for groups D and E were repeated, but receiving or not the inhibitor of iNOS activity L-N6-(1-iminoethyl)-lysine (L-NIL) in the drinking water at $100 \, mg \, l^{-1}$ (calculated dose:$6.7 \, mg \, kg^{-1} \, day^{-1}$) ($n = 8$/group).

Dynamic infusion cavernosometry
It was performed as described.[11–13] In brief, the basal intracavernosal pressure (ICP) was recorded, and 2 mg papaverine was administered through a cannula into the corpora cavernosa. The ICP was recorded 5 min later as the 'ICP after papaverine'. After complete detumescence, saline was infused through another cannula, increasing the infusion rate by $0.05 \, ml \, min^{-1}$ every 10 s, until the ICP reached $100 \, mm \, Hg$ ('infusion rate'). Then the infusion was adjusted to hold the ICP around $100 \, mm \, Hg$ ('maintenance rate'). The 'drop rate' was determined by recording the decrease in ICP within the next 1 min after the infusion was stopped.

Histochemistry and immunohistochemistry
After cavernosometry, the rats were killed and the middle regions of the skin-denuded penile shafts were fixed overnight in 10% formalin, washed and stored in 70% alcohol at $4 \, °C$ until processed for paraffin-embedded tissue sectioning ($5 \, \mu m$). Adjacent sections were used for Masson's trichrome staining for collagen (blue) and SM (red); picrosirius red under polarized microscopy for collagen III (green and green-yellow)/I (red and orange) ratios; and immuno-detection with monoclonal antibodies against α-smooth muscle-actin (ASMA) as an SMC marker (Sigma Kit, Sigma Diagnostics, St Louis, MO, USA), proliferating cell nuclear antigen (PCNA) as a marker of cell proliferation (Chemicon, Temecula, CA, USA), and polyclonal antibodies against transforming growth factor β1 (TGFβ1) (1:200; Promega, Madison, WI, USA) and iNOS (1:500; Calbiochem, La Jolla, CA, USA).[12,37] The specificity of the antibodies was validated by western blot.

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Sections were then incubated with biotinylated anti-mouse immunoglobulin G (IgG) for ASMA and PCNA or biotinylated anti-rabbit IgG for iNOS and TGFβ1, followed by avidin–biotin complex (Vector Labs, Burlingame, CA, USA) and 3,3′ diaminobenzidine (Sigma Chemical, St Louis, MO, USA) for PCNA and iNOS, or the ASMA Sigma Kit for ASMA and 3-amino-9-ethylcarbazole. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay was performed with the Apoptag peroxidase detection assay (Chemicon). The sections were counterstained with hematoxylin. The negative controls for immunohistochemistry were performed by replacing the first antibody with the IgG isotype. For the negative control for the TUNEL assay, buffer was substituted for the terminal deoxynucleotidyl transferase enzyme. Testicular sections from old animals were used as a positive control for TUNEL.

Quantitative image analysis
It was performed by computerized densitometry using the ImagePro Plus, version 5.1, program (Media Cybernetics, Silver Spring, MD, USA) coupled to an Olympus BHS microscope equipped with an Olympus digital camera.[12,13] For Masson staining, ×40 magnification pictures of the penis composed of one half of the corpora cavernosa but excluding the sinusoidal spaces were analyzed for SM (stained in red) and collagen (stained in blue) and expressed as the SM/collagen ratio. An identical approach was used for the collagen III/I ratios. For ASMA and iNOS staining, only the corpora cavernosa was analyzed in a computerized grid and expressed as the percentage of positive area versus total area of the corpora cavernosa. The intensity of immunostaining was determined as the percentage of integrated optical density in the corpora cavernosa. For the TGFβ1, PCNA and TUNEL determinations, the number of positive cells at ×400 was counted, and the results are expressed as the percentage of positive cells/total cells in the corpora cavernosa. In all cases, two fields at ×40, or eight fields at ×400, were analyzed per tissue section, with at least four matched sections per animal and 6–11 animals per group.

Quantitative western blots
Penile homogenates of frozen tissue (100 mg) were obtained in T-PER (PIERCE, Rockford, IL, USA) and protease inhibitors (3 μM leupeptin, 1 μM pepstatin A, 1 mM phenyl methyl sulfonyl fluoride), and centrifuged at 10 000 g for 5 min. Supernatant protein (30 μg) was run on 7.5 or 10% (ASMA) polyacrylamide gels, and submitted to western blot immunodetection with a monoclonal ASMA IgG (1:1000;Oncogene-Calbiochem, La Jolla, CA, USA),

detecting a 43 kDa band. Membranes were incubated with a secondary polyclonal horse anti-mouse IgG linked to horseradish peroxidase (1:2000; BD Transduction Labs, San Diego, CA, USA), and bands were visualized with luminol (Pierce, Rockford, IL, USA).[12,38] A single positive control was run throughout all gels for each antibody to standardize for variations in exposures and staining intensities. Negative controls were performed omitting the primary antibody. Band intensities were determined by densitometry and corrected by the respective intensities for a housekeeping protein, glyceraldehyde phosphate dehydrogenase, upon reprobing.

Collagen estimation in fresh tissue
As previously described, the tissue was homogenized in saline, hydrolyzed with 2N NaOH for 30 min at 120 °C, followed by the estimation of hydroxyproline by a modification of the Neumann and Logan's reaction using Chloramine T and Ehrlich's reagent, against a hydroxyproline standard curve and measuring at 550 nm.[11–13,38] Values were expressed as μg of collagen per mg of tissue.

Statistical analysis
The values are expressed as the mean ± s.e.m. The normality distribution of the data was established using the Wilk–Shapiro test. Multiple comparisons were analyzed by a single factor analysis of variance, followed by *post hoc* comparisons with the Newman–Keuls test, according to the GraphPad Prism, version 4.1 for windows (GraphPad Software, San Diego CA, USA). Differences were considered significant at $P < 0.05$.

Results

There was no significant difference in body weights between the groups. Sildenafil treatment did not cause priapism, lethargy, aggressiveness or any other noticeable side effect. Rats were subjected to cavernosometry after a 24 h washout period that reduces sildenafil concentrations to baseline.[12,39] Rats undergoing UCNR and BCNR for 45 days had a significant increase in the drop rate as compared to sham-operated animals, which was higher after BCNR than UCNR (Figure 1 bottom), and this was accompanied by a significant reduction of the response to papaverine (top), but only in the BCNR rats. This confirmed our previous observation that CVOD is induced by BCNR, and showed that UCNR induced a moderate CVOD.[14] Continuous oral treatment with sildenafil normalized the drop rate and response to papaverine (top and bottom) in both the UCNR and BCNR animals.
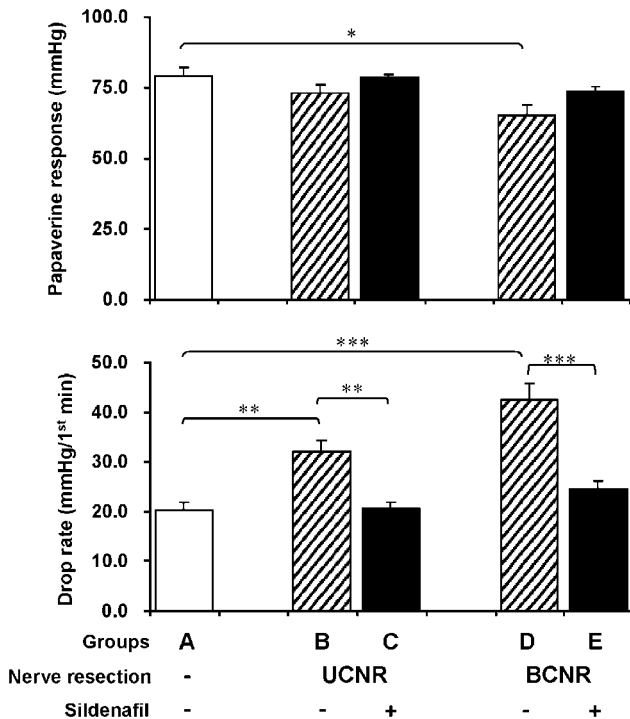
Figure 1 Effect of unilateral and bilateral cavernosal nerve resection (BCNR) and long-term sildenafil treatment on the erectile function of the rat measured by pharmacological and infusion cavernosometry. Sildenafil treatment was given for 45 days after nerve resection. Top: Response of the intracavernosal pressure to papaverine; Bottom: Response of the intracavernosal pressure to the interruption of saline infusion. A: sham-operated rats; B: untreated unilateral cavernosal nerve resection (UCNR); C: UCNR with sildenafil; D: untreated BCNR; E: BCNR with sildenafil. $*P<0.05$; $**P<0.01$; $***P<0.001$.

The underlying changes in corporal histology reflected the cavernosometric findings in terms of the smooth muscle/collagen ratio, and SMC content as shown on the representative microphotographs of cross sections of the corpora cavernosa obtained from sham, BCNR untreated and BCNR treated rats, and stained by Masson trichrome or ASMA (Figure 2 top panels). The estimations were performed around the lacunar spaces area where the SMC are concentrated. Both BCNR and UCNR reduced the SMC to collagen ratio, as estimated by Masson trichrome and quantitative image analysis, by about 30% as compared to sham-operated rats, and this was prevented by sildenafil treatment in both the BCNR and UCNR animals (Figure 2 top graph). That these changes in the ratio were due, at least partially, to absolute changes in the SMC content, was shown by quantitative immunohistochemistry for ASMA as a marker of SMC (Figure 2 bottom graph). Both UCNR and BCNR reduced this SMC content by about 60%, at least directly around the lacunar spaces, and sildenafil increased it significantly, but did not fully normalize it.

SMC content was also evaluated in total corpora cavernosa homogenates of the BCNR specimens only, via ASMA immunodetection by quantitative western blot. These preparations of the homogenates include, in addition to corporal SMC, the SMC from the corpus spongiosum and the media of the corporal arteries as well as potential myofibroblasts in the tunica or interstitial connective tissue, whereas the immunohistochemical detection performed above is restricted to corporal SMC. The ASMA band was considerably increased by sildenafil treatment, in comparison to the untreated BCNR rats (Figure 3 top), and the densitometric quantitation (Figure 3 bottom) indicates an over 2-fold increase which agrees with what was observed in the tissue sections.

Collagen content and composition were both affected by nerve resection and by sildenafil treatment. The collagen content was significantly increased by BCNR, but not by UCNR when compared to the sham-operated animals (Figure 4 bottom graph). Continuous long-term sildenafil treatment reduced the collagen content in the BCNR rats, but did not normalize this value. Both BCNR and UCNR decreased the collagen III/I ratio (Figure 4 top graph) as seen previously with vardenafil, although in the UCNR group, this did not reach statistical significance. Sildenafil normalized the collagen III/I ratio in the BCNR rats.[33]

The reduction in SMC was accompanied by a nearly 3-fold increase in apoptosis induced by BCNR and UCNR (Figure 5 top graph), when compared to the sham-operated animals. The % apoptotic index was reduced to normal values in both BCNR and UCNR with continuous long-term sildenafil treatment. The levels of TGFβ1, presumably involved in collagen deposition and SMC loss, were also increased by nearly 2-fold by both UCNR and BCNR (Figure 5 bottom graph), and restored to sham values with continuous long-term sildenafil treatment.

Cell replication within the corpora was evaluated by staining for PCNA (Figure 6 top graph), showing that the % proliferation index was reduced 3–4-fold by UCNR and BCNR compared to sham-operated animals. Continuous long-term sildenafil treatment stimulated cell proliferation restoring the low values seen in the UCNR and BCNR groups back up to the values of the sham controls. As a result, the ratio between the cell proliferation and apoptosis, an indicator of cell turnover, was dramatically reduced by both UCNR and BCNR, and while this was counteracted by sildenafil, normal values were only restored in the UCNR animals.

iNOS, a putative anti-fibrotic and pro-apoptotic factor, was increased 3–4-fold in both the UCNR and BCNR rats when compared to the control animals, as shown by immunohistochemistry (Figure 7). However, in the long-term sildenafil treated animals, which demonstrated a decrease in both fibrosis and apoptosis when compared to the non-sildenafil treated UCNR and BCNR animals, iNOS induction

Figure 2 Effect of unilateral and bilateral cavernosal nerve resection and long-term sildenafil treatment on the smooth muscle/collagen ratio and smooth muscle cell content in the rat corpora cavernosa. Tissue sections from the experimental rat groups in Figure 1 were submitted to Masson's trichrome staining (Top micrographs), and other adjacent sections were immunostained for α-smooth muscle-actin (ASMA) as a smooth muscle cell marker (bottom micrographs). The micrographs depict representative fields ($\times 200$, bar $= 200\,\mu$m) and the bar plots show the respective quantitative image analysis. A: sham-operated rats; B: untreated unilateral cavernosal nerve resection (UCNR); C: UCNR with sildenafil; D: untreated bilateral cavernosal nerve resection (BCNR); E: BCNR with sildenafil. $*P<0.05$; $**P<0.01$; $***P<0.001$.

was not altered and remained elevated like those of the untreated UCNR and BCNR animals.

To determine whether iNOS spontaneous induction acts as an antifibrotic mechanism subsequent to cavernosal nerve damage, and whether it plays any role in the protection exerted by sildenafil, L-NIL was given to new groups of BCNR rats immediately after the intervention, and these animals were treated or not with sildenafil for 45 days. Other two similar groups did not receive L-NIL. Cavernosometry showed that in the BCNR rats the ICP after papaverine was reduced by L-NIL treatment to the very low value (in mm Hg) of 23.0 ± 9.7 as compared to 62.9 ± 9.8 in the untreated controls ($P<0.001$). However, L-NIL did not reduce significantly the ICP in the BCNR rats treated with both L-NIL and sildenafil (65.9 ± 13.3) compared with the sildenafil only treated BCNR rats (69.5 ± 10.8).

iNOS long-term inhibition caused also a significant reduction of the SMC/collagen ratio in the sildenafil-untreated BCNR rats, to 0.05 ± 0.003 from 0.09 ± 0.006 in the control ($P<0.05$), in good

agreement to the effects of L-NIL on the erectile response to papaverine. However, despite the lack of significant inhibition on the functional effects of sildenafil, L-NIL moderately interfered with sildenafil effects on the SMC/collagen ratio, by reducing this value from 0.15 ± 0.01 to 0.10 ± 0.006 ($P<0.001$).

Discussion

These results confirm and extend our previous work with vardenafil in BCNR rats by showing that UCNR is *per se* deleterious to the corpora tissue, resulting in a reduction in the SMC content, and SMC proliferation, while increasing collagen content, TGFβ1, apoptosis and iNOS induction, to nearly the same extent as BCNR.[33] In addition, the only parameters that UCNR affected significantly less than BCNR were CVOD and collagen deposition thereby suggesting that a certain level of collagen
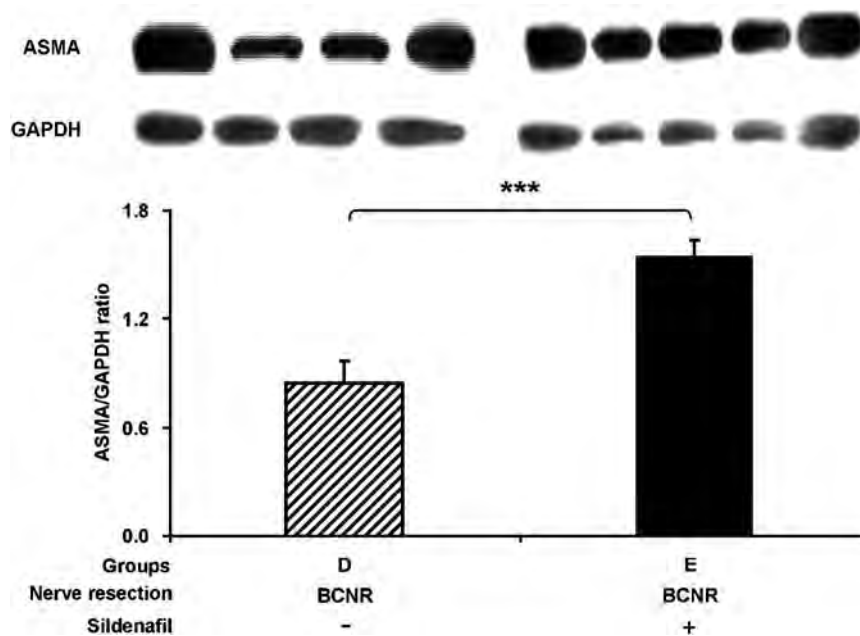
Figure 3 Effect of long-term sildenafil treatment on the expression of α-smooth muscle-actin (ASMA) in total penile shaft tissue from rats subjected to bilateral cavernosal nerve resection. Homogenates from total penile shaft tissue were submitted to western blot for ASMA. Top: representative pictures of the gels depicting the ASMA band. Bottom: densitometric analysis. ***$P < 0.001$.
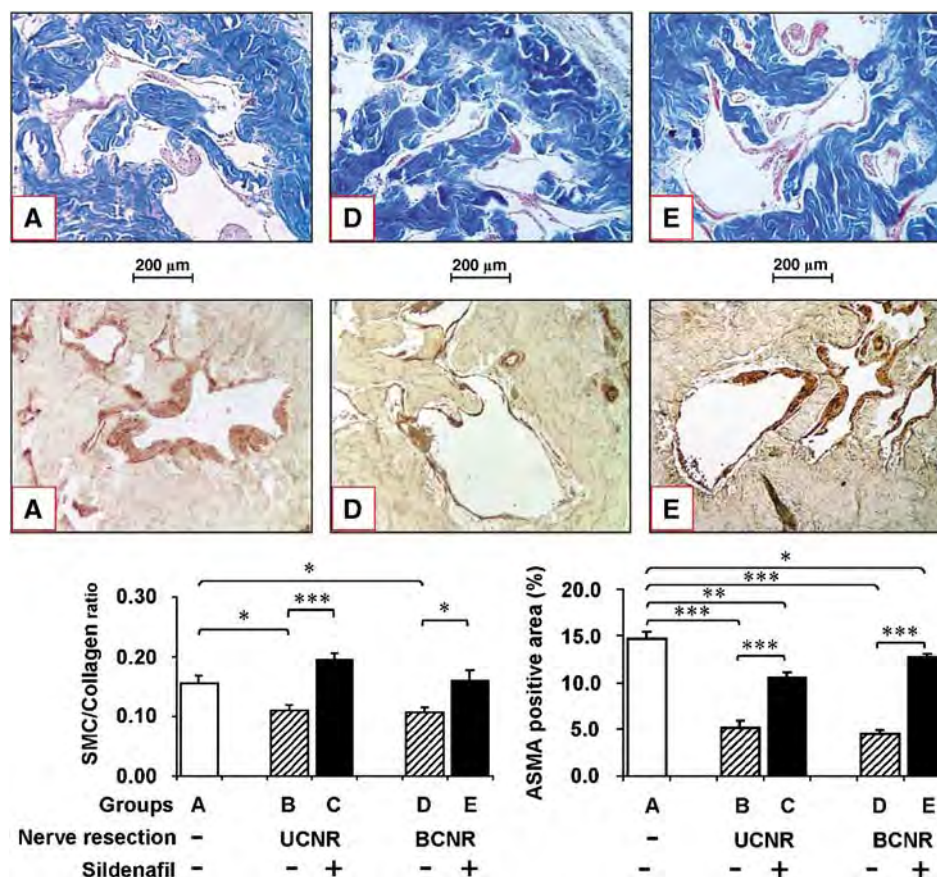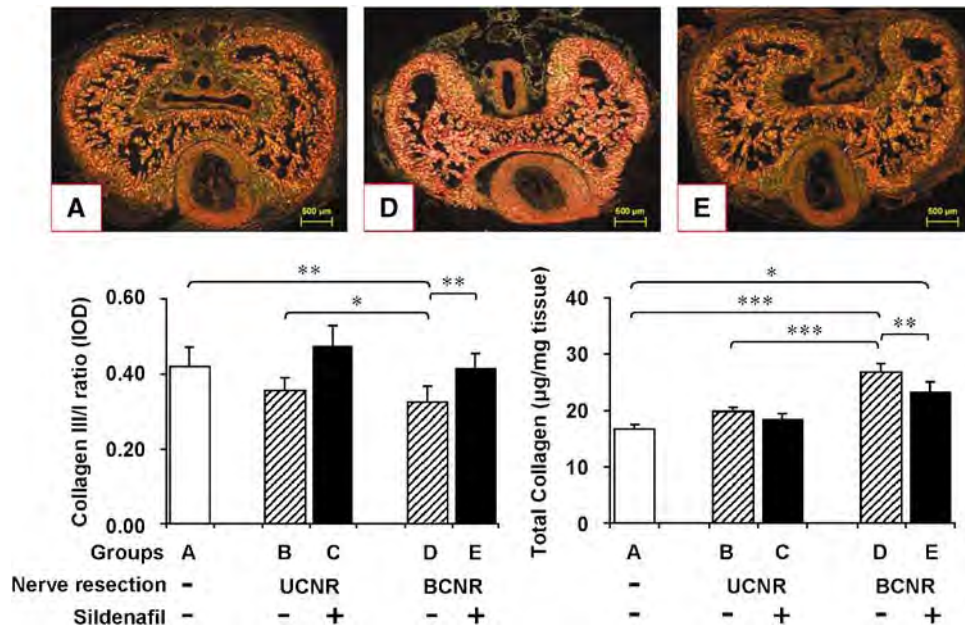

Figure 4 Effect of unilateral and bilateral cavernosal nerve resection and long-term sildenafil treatment on the collagen III/I ratio and total collagen content in the rat corpora cavernosa. Adjacent tissue sections to those in Figure 2 were submitted to Picro-Sirius red staining and visualized under polarized light (top micrographs). Frozen penile specimens were used to determine the total collagen content by a hydroxyproline assay (bottom). The micrographs depict representative fields taken at ×40 with the method of overlapping fields and assembled to represent the whole penis (bar: 500 μm) to show the distribution of collagen I (red/orange) and collagen III (green–greenish-yellow), and the bar plots show the quantitative image analysis. A: sham-operated rats; B: untreated unilateral cavernosal nerve resection (UCNR); C: UCNR with sildenafil; D: untreated bilateral cavernosal nerve resection (BCNR); E: BCNR with sildenafil. *$P < 0.05$; **$P < 0.01$ ***$P < 0.001$.

deposition may be necessary to cause a frank CVOD. The PCNA results confirmed our previous contention that there is a basal SMC proliferation occurring in the corpora cavernosa, similarly to what occurs in the arterial media, which is reduced by cavernosal nerve damage.[40] Furthermore, oral continuous
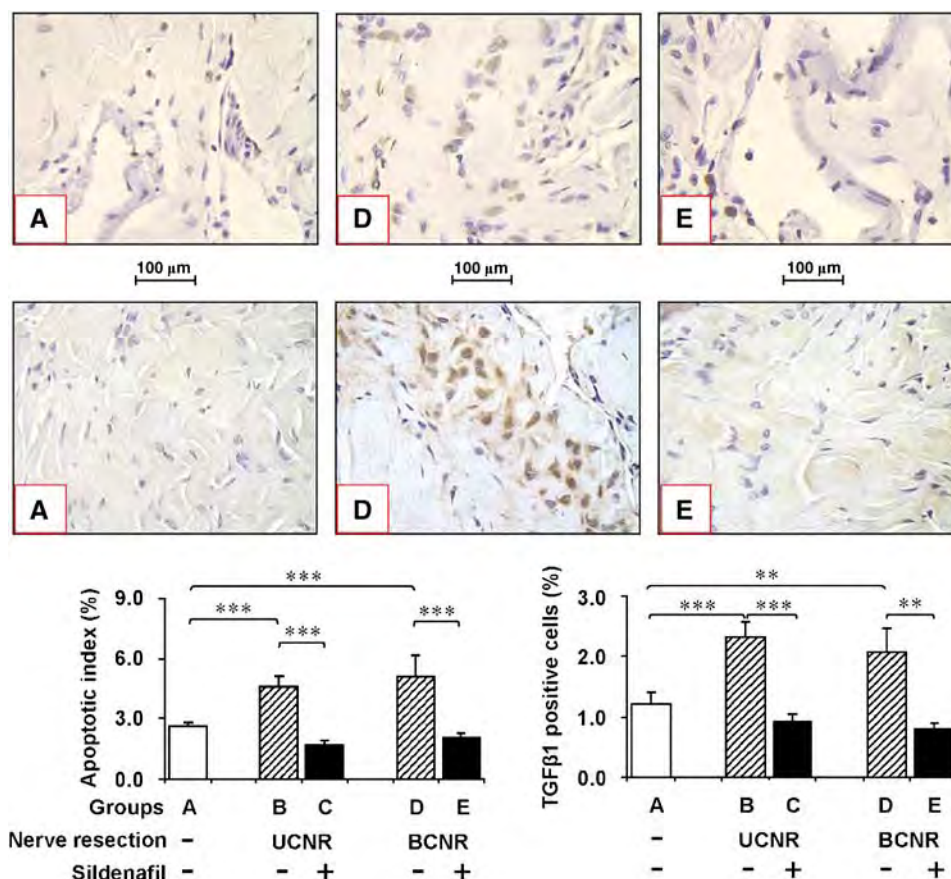
Figure 5 Effect of unilateral and bilateral cavernosal nerve resection and long-term sildenafil treatment on apoptosis and TGFβ1 expression in the rat corpora cavernosa. Adjacent tissue sections to those in the preceding figures were submitted to TUNEL staining (top micrographs), and other sections were immunostained for TGFβ1 (bottom micrographs). The micrographs depict representative fields ($\times 400$, bar $= 100 \mu$m) and the bar plots show the quantitative image analysis. A: sham-operated rats; B: untreated unilateral cavernosal nerve resection (UCNR); C: UCNR with sildenafil; D: untreated bilateral cavernosal nerve resection (BCNR); E: BCNR with sildenafil. $^{**}P < 0.01$; $^{***}P < 0.001$.

long-term treatment with sildenafil, as opposed to the sporadic on demand treatment that is used to elicit an erection, normalized the physiological and for the most part the tissue composition values in the BCNR rats. And finally, as seen with vardenafil, the continuous long-term treatment with sildenafil did not cause noticeable adverse side effects. Moreover, a prolonged treatment with sildenafil in the rat does not induce tachyphylaxis by PDE5 upregulation.[41]

Because UCNR and BCNR represent from an experimental point of view what may result from either a partial or complete cavernosal nerve injury, respectively, during a radical prostatectomy, these observations together with those previously shown with vardenafil support the continuous use of oral PDE5 inhibitors post-prostatectomy for preventing the histological changes in the corpora that CVOD and resultant erectile dysfunction. The dose that we have used in this present study is likely to be considered excessive for routine clinical use, since it is roughly equivalent to 200 mg day^{-1} for men.[12,38] However, we chose this dose based on a parallel

study in the aged rat where sildenafil was given in a similar continuous and long-term manner to prevent the histological and physiological changes associated with aging related erectile dysfunction which has been shown to be primarily due to CVOD.[12]

This 20 mg kg^{-1} day^{-1} dose of sildenafil in the rat is about 10-fold higher than the one we used for vardenafil in our BCNR rat model and is within the dosages that have been tested in continuous administration in experimental animals and even in patients for conditions such as pulmonary hypertension.[33,42–44] With the exception of the anti-apoptotic effect that we found with sildenafil in the current study which we did not see with vardenafil in our previous report, both drugs elicited the same effects despite the difference in dosage.[33] Therefore, it is likely that a lower dose of sildenafil would also be as effective. Obviously, a dose response study should be explored in this experimental nerve resection model using retrolingual daily doses, although the pharmacokinetics of sildenafil clearance may require a higher dose.[37,39]
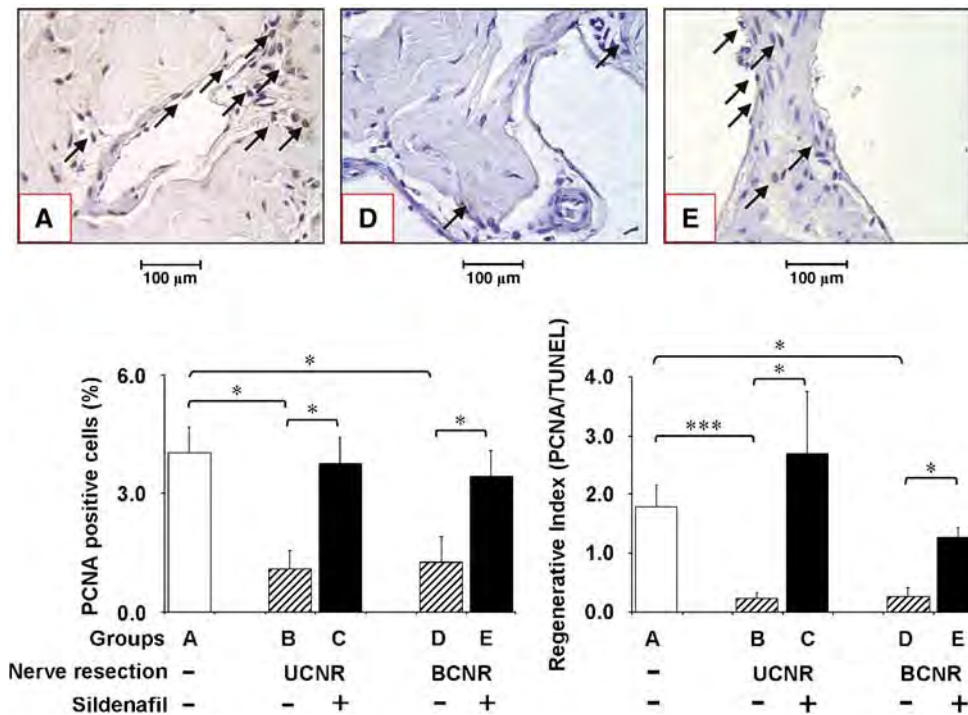
Figure 6 Effect of unilateral and bilateral cavernosal nerve resection and long-term sildenafil treatment on cell proliferation and turnover in the rat corpora cavernosa. Adjacent tissue sections to those in the preceding figures were submitted to PCNA immuno-staining (top micrographs), and the bar plots show the quantitative image analysis. The micrographs depict representative fields (× 400, bar = 100 μm). The ratio between the proliferation index and the apoptotic index calculated from Figure 4 was plotted (bottom) A: sham-operated rats; B: untreated unilateral cavernosal nerve resection (UCNR); C: UCNR with sildenafil; D: untreated bilateral cavernosal nerve resection (BCNR); E: BCNR with sildenafil. *P<0.05; ***P<0.001.
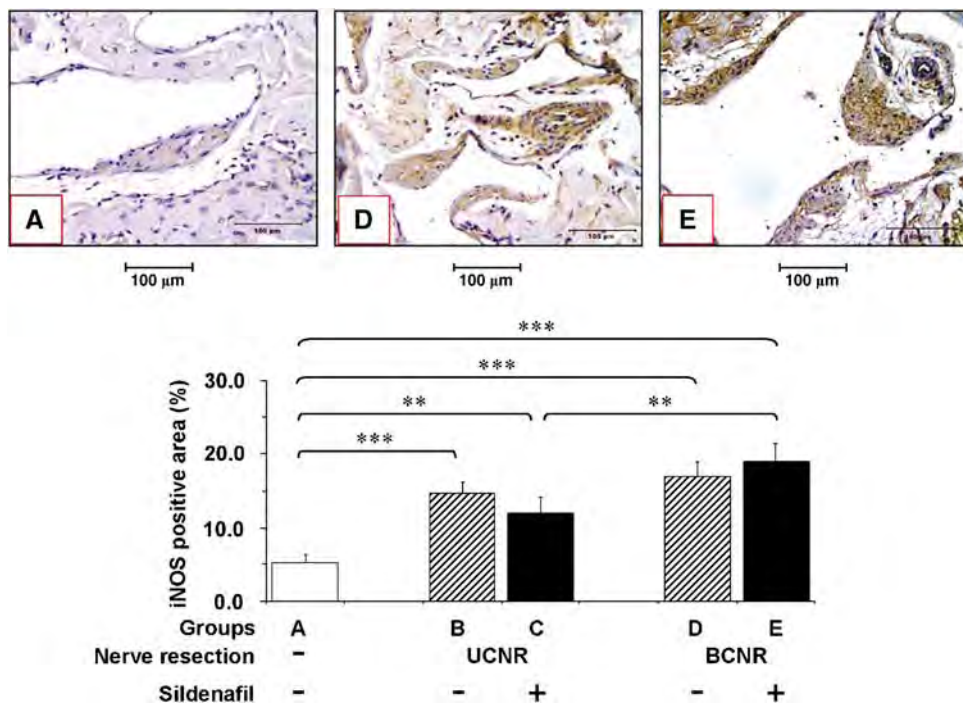
Figure 7 Effect of unilateral and bilateral cavernosal nerve resection and long-term sildenafil treatment on inducible nitric oxide synthase (iNOS) expression in the rat corpora cavernosa. Adjacent tissue sections to those in the preceding figures were submitted to iNOS immuno-staining. The micrographs depict representative fields (× 400, bar = 100 μm) and the bar plots show the quantitative image analysis. A: sham-operated rats; B: untreated unilateral cavernosal nerve resection (UCNR); C: UCNR with sildenafil; D: untreated bilateral cavernosal nerve resection (BCNR); E: BCNR with sildenafil. **P<0.01; ***P<0.001.

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Based on these current findings in the UCNR and BCNR models with sildenafil and those previously with vardenafil in the BCNR model, together with that observed in the aged rat treated with sildenafil, we believe that the PDE5 inhibitors possess anti-fibrotic activity which in the penile tissue may lead to the prevention or delay in the onset or progression of CVOD regardless of its etiology.[33,12] Although cGMP was not estimated in the penile tissue in this study because the rats were killed after a 24 h washout, we assume that the high cGMP levels generated by PDE5 inhibitors are what counteracts fibrosis and protects the corporal smooth muscle.[12,33,37,38] This assumption is based on the known inhibitory effects of cGMP on collagen synthesis and the TGFβ1 pathway, and its vasculo-protective effects on arterial SMC.[14–17,20,45–47] However, the anti-apoptotic and pro-proliferative effects of sildenafil that we found in the corporal smooth muscle do not agree with the fact that cGMP inhibits vascular SMC proliferation.[48,49] Since the effects of BCNR on the corporal histology and pharmacokinetics are the same as those seen in the aged rat, we assume that cGMP effects on corporal SMC may be modulated by some specific features in the corporal SMC themselves or the tissue milieu, that are not operative in the vascular SMC.[12]

Another still unresolved question is the role of iNOS induction in corporal atrophy after cavernosal nerve damage. Studies on liver and kidney fibrosis in the iNOS knock-out mouse, and our previous work in Peyronie's disease and its animal models, vaginal fibrosis, aging-related arterial media fibrosis, and in corporal fibrosis, suggests that despite iNOS may be initially induced during an early inflammatory process, its main role is as an antifibrotic agent.[12,13,24–29,32,33,37,38,50] This may occur via cGMP produced by the nitric oxide from iNOS, and/or the nitric oxide can directly reduce collagen synthesis, myofibroblast formation in the interstitial connective tissue, and reactive oxygen species.[26,30–32] In contrast to our study with vardenafil in the BCNR rat, the slight stimulation of iNOS induction by sildenafil in the BCNR animals was not significant.

It is noteworthy that the high iNOS levels in the presence of sildenafil did not counteract the normalization of the apoptotic index or the upregulation of cell replication exerted by the drug. Since nitric oxide in the vasculature is usually pro-apoptotic and antiproliferative, this would support our assumption regarding the opposite response of corporal and arterial SMC toward the nitric oxide/cGMP pathway, in this respect. The experiment using long-term continuous L-NIL as an iNOS inhibitor supported the view that iNOS acts as a true endogenous antifibrotic agent in the BCNR model, as it was in the aged rat.[29] However, the protective effects of long-term continuous sildenafil on restoring erectile function and reducing corporal fibrosis in this model do not seem be mainly mediated by iNOS induction,

since L-NIL did not significantly affect the functional response to sildenafil, and interfered only partially with the improvement of the SMC/collagen ratio exerted by the PDE5 inhibitor.

We acknowledge that since this work, as well as the preceding one with vardenafil, has focused on SMC number and fibrosis, we cannot exclude that sildenafil action may also involve protecting the SMC relaxation/contractile phenotype and/or the integrity of the cavernosal endothelium, but we believe the intracellular/extracellular composition of the corporal smooth muscle is the main target of this drug when given long-term, since this balance is responsible for tissue compliance to relaxation by nitric oxide.[33,51–55]

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Long-term, continuous administration of PDE5 inhibitors alone or combined with implantation of stem cells in the penile corpora cavernosa, prevents erectile dysfunction in a rat model of cavernosal nerve damage after radical prostatectomy

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BACKGROUND

- Erectile dysfunction (ED) is a major risk of radical prostatectomy (RP) for prostate cancer and a deterrent for many patients to choose this therapy.
- The neuropraxia due to cavernosal nerve damage causes the most prevalent vasculogenic ED (corporal veno-occlusive dysfunction (CVOD) or venous leakage). In CVOD blood cannot be efficiently retained in the penis to achieve a rigid erection.
- CVOD occurs by the impairment of penile corpora cavernosa relaxation by the loss/damage of smooth muscle (SM) cells (atrophy) and excessive collagen deposition (fibrosis). Cavernosal nerve damage leading to ED is reduced but not eliminated by the use of bilateral nerve sparing surgery, and in any case, practice of this technique is restricted to certain centers of excellence
- We showed in rat models that fibrosis of the corpora cavernosa is accompanied by the induction of inducible nitric oxide synthase (iNOS), and that gene therapy with iNOS cDNA, or a chronic administration of nitric oxide (NO) generators or phosphodiesterase 5 inhibitors (PDE5i), such as si ldenafil, reduces the corporal fibrosis and SM loss underlying CVOD associated with aging, as well as the penile tunical fibrosis in the localized plaque that characterizes Peyronie's disease.
- The main mediator of this endogenously elicited mechanism of defense against fibrosis is the synthesis in the corpora cavernosa of high levels of NO and/or its product, cGMP, maintained during a period long enough as to counteract a chronic process
- *This cannot be achieved by the current clinical administration of PDE5i "on demand"*, where the peaks of corporal NO and/or cGMP levels are limited by the pharmacokinetics of each drug, and the duration of sexual stimulation. *The current modality of PDE5i administration is merely palliative and not curative of the corporal histopathology that underlies CVOD.*
- We showed a similar correction of the corporal histopathology by implanting adult stem cells into the corpora cavernosa of aged rats, reversing the ED associated with aging. We have used skeletal muscle derived stem cells (MDSC) and demonstrated that they can originate SMC.
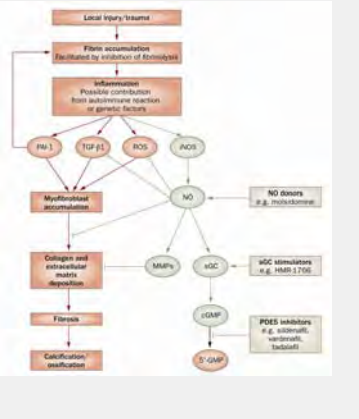
Figure 1
Proposed mechanisms for the antifibrotic and smooth muscle protective effects of a steady long-term stimulation of the levels of NO and cGMP in the corpora cavernosa. The experimental work described in the subsequent figures has validated our hypothesis but this needs to be tested in clinical trials.

OBJECTIVES

We aimed to investigate in a rat model of cavernosal nerve damage after RP based on unilateral (UCNR) and bilateral (BCNR) cavernosal nerve resection:
- whether CVOD caused by RP may be prevented by a long-term continuous administration of PDE5i and/or NO generators, in combination or not with MDSC, to protect corporal SM and avoid fibrosis; and
- the role played by iNOS in this process.

ANIMAL TREATMENTS

FOR PUBLISHED STUDIES SUPPORTED BY THIS GRANT
Surgery and treatments: Nerve resection was performed by resecting the cavernosal nerves by removing a 3-mm segment uni- or bilaterally. In the sham-operated group, both cavernosal nerves were identified but not resected. Actual drug doses are expressed as mg/kg/day, with the approximate equivalent dose in men as mg/day (bold).
Animals: Five-month-old male Fisher 344 rats were treated and divided as follows: Sildenafil at high dose (n=10/group): A) sham-operated; B) UCNR, C) UCNR + sildenafil: 20 (200), D) BCNR and E) BCNR + sildenafil: 20 (200). Sildenafil was dissolved in the drinking water and given for 45 days. Washout was 1 day. In a subsequent experiment, treatments for groups D and E were repeated (n=8/group), but receiving or not the inhibitor of iNOS activity L-NIL in the water (6.7)
Tadalafil at high dose (n=10/group): A) s ham-operated; B) UCNR, C) UCNR + tadalafil: 5 (50), D) BCNR, and E) BCNR + tadalafil: 5 (50). Tadalafil was dissolved in 10% glucose + 1% Tween 80 and administered retrolingually once per day. Washout was 3 days.
Time course of events in untreated animals (n=8/group): A) sham operated and B) BCNR groups, left untreated, and sacrificed at 1, 3, 7, 15, 30, and 45 days after surgery.
FOR CURRENT STUDY (PAPER IN PREPARATION)
Sildenafil at lower doses, with or without NO donors (n=8/group): 1) Sham; 2) BCNR untreated; 3) BCNR, sildenafil medium dose, in water: 10 (100); 4) BCNR, sildenafil low dose, in water: 2.5 (25); 5) BCNR, sildenafil low dose, retrolingual: 2.5 (25) 6) BCNR, molsidomine IP 10 (100), 7) BCNR, molsidomine IP 10 (100), with sildenafil medium dose in water 10 (100); and 8) BCNR, molsidomine IP 10 (100), with sildenafil low dose 2.5 (25).
Sildenafil at very low dose with or without MDSC implantation (n=8/group): 9) sildenafil, very low dose, in water: 1.25 (12.5); 10) sildenafil, very low dose in water: 1.25 (12.5) + MDSC (10⁶ cells), intracorporeal; 11) MDSC (10⁶ cells) alone, intracorporeal.
Outcome methods: Dynamic Infusion Cavernosometry (DIC) was applied for measuring CVOD. Quantitative Masson trichrome and immunohistochemical staining in corporal tissue sections for αSMA (alpha smooth muscle actin), apoptosis, proliferating cell nuclear antigen (PCNA), iNOS, and nNOS, was applied, confirming by quantitative western blots. Total collagen was estimated by hydroxyproline assay.
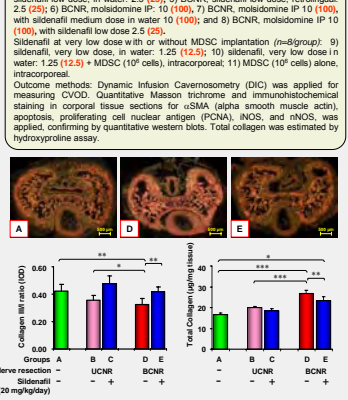
Figure 2
Effect of unilateral and bilateral cavernosal nerve resection and long-term sildenafil treatment on the collagen III/I ratio and total collagen content in the rat corpora cavernosa. Picro-Sirius red staining visualized under polarized light. Top: Representative micrographs show the distribution of collagen I (red/orange) and collagen III (green–greenish-yellow), (X40). Bottom left: Qualitative image analysis Frozen penile specimens were used to determine the total collagen content by a hydroxyproline assay (bottom). the bar plots show the quantitative image analysis. A: sham-operated rats; B: untreated UCNR; C: UCNR with sildenafil; D: untreated BCNR; E: BCNR with sildenafil. *P<0.05, **P<0.01; ***P<0.001.
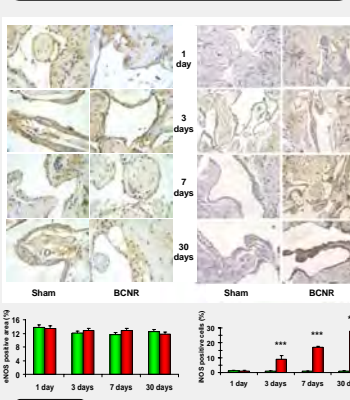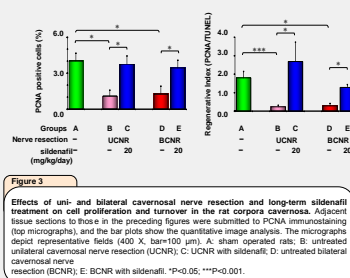
Figure 3
Effects of uni- and bilateral cavernosal nerve resection and long-term sildenafil treatment on cell proliferation and turnover in the rat corpora cavernosa. Adjacent tissue sections to those in the preceding figures were submitted to PCNA immunostaining (top micrographs), and the bar plots show the quantitative image analysis. The micrographs depict representative fields (400 X, bar=100 μm). A: sham operated rats; B: untreated unilateral cavernosal nerve resection (UCNR); C: UCNR with sildenafil; D: untreated bilateral cavernosal nerve resection (BCNR); E: BCNR with sildenafil. *P<0.05; ***P<0.001.

Figure 4
Time course of the effect of BCNR on the expression of endothelial - (eNOS) and inducible nitric oxide synthase (iNOS) in the rat corpora cavernosa. Rats were killed at 1, 3, 7, and 30 days after either sham or nerve resection operation. Paraffin embedded penile cross sections were immunostained against eNOS and iNOS. The expression of eN OS is not altered by n erve resection. iNOS over expression started increase by 10-fold at day 3, and continued to remain high throughout the study period, while it stayed almost undetectable in the sham-operated animals at all time periods. Left column: eNOS data. Right column: iNOS data. Top: r epresentative photomicrographs (X200). Bottom: quantitative image analysis. ***P < 0.001.

Figure 5
Effects of long-term sustained sildenafil treatment in lower doses on BCNR induced CVOD and smooth muscle loss/fibrosis with and without molsidomine and MDSC.
Left: Drop rate of intracavernosal pressure measured by dynamic infusion cavernosometry. Right: Smooth muscle /collagen ratio changes assessed by Masson trichrome staining followed by a quantitative image analysis. All treatment are normalized the high drop rate, however the low dose (2.5 mg/kg/day) retrolingual (RL) and the very low dose (1.5 mg/kg/day) sildenafil were not able to prevent the decrease of the SMC/collagen ratio. *P<0.05; **P<0.01; *** P < 0.001.
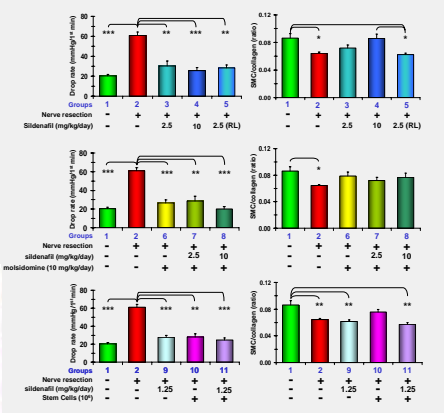
GENERAL INTEGRATION OF OUR RESULTS

Time course
- Following BCNR, CVOD slowly developed and was detectable at 30 days, and it becoming more pronounced by 45 days.
- In contrast, the SMC/collagen ratio in the BCNR corpora was reduced at 7 days and bottomed at 30 and 45 days, due in part to the reduction of SMC, presumably caused by an increase in apoptosis peaking at 3 days. PCNA also peaked at 3 days, but then decayed.
- nNOS was reduced early (3–7 days) and disappeared at 30 days, whereas eNOS was not affected. iNOS was induced at day 3, and steadily increased peaking at 30 days.
Effects of continuous long term treatment with sildenafil and tadalafil at high doses
- Sildenafil normalized at a high dose equivalent to 200 mg in men, the CVOD, measured by dynamic infusion cavernosometry, induced by both BCNR and UCNR.
- The drug prevented the 30% decrease in the penile corporal smooth muscle cell/collagen ratio, and the 3-4-fold increase in apoptosis and reduction in cell proliferation, and partially counteracted the increase in collagen, seen with both UCNR and BCNR;
- The long-term inhibition of iNOS activity exacerbated corporal fibrosis and CVOD in the BCNR rats, but sildenafil functional effects were not affected by L-NIL.
- Tadalafil long-term treatment exerted similar effects
Effects of continuous long term treatment with sildenafil at lower doses with or without molsidomine, and MDSC with or without sildenafil at very low dose
- All treatments normalized the high drop rate in the dynamic infusion and improved the response to intracorporeal papaverine.
- The SMC/collagen ratio was significantly increased but not completely normalized by treatment in all series.

CONCLUSIONS

- Our pre-clinical experimental studies support the concept that vasculogenic ED results from cavernosal neuropraxia during RP, causing the early loss of corporal SMC by apoptosis, which the initial cell replication response cannot counteract, followed by fibrosis. The time course of iNOS induction supports the antifibrotic role of iNOS
- An early long-term continuous treatment with PDE5i, even at low doses, as opposed to current on demand regimens, may prevent CVOD through correction of the underlying corporal histopathology.
- We also propose that the intracorporeal implantation of stem cells may facilitate corporal tissue repair and prevent CVOD subsequent to RP, but further studies are needed to optimize the cell type, régimen of implantation, and the combination or not with PDE5i

IMPACT ON THE FIELD

These results provide scientific bases for the emerging clinical interest in preventing or "curing" ED with PDE5i, rather than merely using them palliatively to facilitate an erection and for pharmacological "penile rehabilitation" after RP.

CLINICAL FOLLOW UP

- Tadalafil is approved for daily use and both 5 and 2.5 mg tablets appear to be effective in a 14 day treatment (Seftel et al, J Urol 2011, 185:243-48), although longer studies are needed to establish curative effects
- Nightly sildenafil (50 mg) for 9 months one month after RP enhance penile recovery (McCullough AR, J Urol 2010, 183:2451-6)

OUR MOST RELEVANT BIBLIOGRAPHY

PDE5 inhibitors/cavernosal nerve damage/penile fibrosis
- *Mechanisms of penile fibrosis.* Gonzalez-Cadavid NF. J Sex Med. 2009 Mar;6 Suppl 3:353-62.
- *Fibrosis and loss of smooth muscle in the corpora cavernosa precede corporal veno-occlusive dysfunction (CVOD) induced by experimental cavernosal nerve damage in the rat.* Ferrini MG, Kovanecz I, Sanchez S, Umeh C, Rajfer J, Gonzalez-Cadavid NF. J Sex Med. 2009 Feb;6(2):415-28.
- *Chronic daily tadalafil prevents the corporal fibrosis and veno-occlusive dysfunction that occurs after cavernosal nerve resection.* Kovanecz I, Rambhatla A, Ferrini MG, Vernet D, Sanchez S, Rajfer J, Gonzalez-Cadavid N. BJU Int. 2008 Jan;101(2):203-10.
- *Long-term continuous sildenafil treatment ameliorates corporal veno-occlusive dysfunction (CVOD) induced by cavernosal nerve resection in rats.* Kovanecz I, Rambhatla A, Ferrini M, Vernet D, Sanchez S, Rajfer J, Gonzalez-Cadavid N. Int J Impot Res. 2008 Mar-Apr;20(2):202-12.
- *Rationale for phosphodiesterase 5 inhibitor use post-radical prostatectomy: experimental and clinical review.* Rambhatla A, Kovanecz I, Ferrini M, Gonzalez-Cadavid NF, Rajfer J. Int J Impot Res. 2008 Jan-Feb;20(1):30-4.
- *Vardenafil prevents fibrosis and loss of corporal smooth muscle that occurs after bilateral cavernosal nerve resection in the rat.* Ferrini MG, Davila HH, Kovanecz I, Sanchez SP, Gonzalez-Cadavid NF, Rajfer J. Urology. 2006 Aug;68(2):429-35.
- *Long-term continuous treatment with sildenafil ameliorates aging-related erectile dysfunction and the underlying corporal fibrosis in the rat.* Kovanecz I, Ferrini MG, Vernet D, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. Biol Reprod. 2007 May;76(5):915-23.
Stem cells/urological fibrosis
- *Stimulating vaginal repair in rats through skeletal muscle-derived stem cells seeded on small intestinal submucosal scaffolds.* Ho MH, Heydarkhan S, Vernet D, Kovanecz I, Ferrini MG, Bhatia NN, Gonzalez-Cadavid NF. Obstet Gynecol. 2009 Aug;114(2 Pt 1):300-9.
- *Effect of muscle-derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat.* Nolazco G, Kovanecz I, Vernet D, Gelfand RA, Tsao J, Ferrini MG, Magee T, Rajfer J, Gonzalez-Cadavid NF. BJU Int. 2008 May;101(9):1156-64.

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Long-term daily low doses of sildenafil, molsidomine, and corporal implantation of muscle derived stem cells (MDSC), alone or in combination, prevent corporal veno-occlusive dysfunction (CVOD) in a rat model of cavernosal nerve damage

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1 Department of Surgery, Division of Urology, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA; 2 Department of Internal Medicine, Charles Drew University of Medicine and Science, Los Angeles, CA; 3 Department of Urology, David Geffen School of Medicine at UCLA, Los Angeles, CA

BACKGROUND

- Erectile dysfunction (ED) is a major risk of radical prostatectomy (RP) for prostate cancer and a deterrent for many patients to choose this therapy.
- The neuropraxia due to cavernosal nerve damage causes the most prevalent vasculogenic ED (cavernosal veno-occlusive dysfunction (CVOD) or venous leakage). In CVOD blood cannot be efficiently retained in the penis to achieve a rigid erection.
- CVOD occurs by the impairment of penile corpora cavernosa relaxation by the loss/damage of smooth muscle (SM) cells (atrophy) and excessive collagen deposition (fibrosis). Cavernosal nerve damage leading to ED is reduced but not eliminated by the use of bilateral nerve sparing surgery, and in any case, practice of this technique is restricted to certain centers of excellence.
- We showed in rat models that fibrosis of the corpora cavernosa is accompanied by the induction of inducible nitric oxide synthase (iNOS), and that gene therapy with iNOS cDNA, or a chronic administration of nitric oxide (NO) generators or phosphodiesterase 5 inhibitors (PDE5i), such as sildenafil, reduces the corporal fibrosis and S M loss underlying CVOD associated with aging, as well as the penile tunical fibrosis in the localized plaque that characterizes Peyronie's disease.
- The main mediator of this endogenously elicited mechanism of defense against fibrosis is the synthesis in the corpora cavernosa of high levels of NO and/or its product, cGMP, maintained during a period long enough as to counteract a chronic process.
- We showed in rats with bilateral cavernosal nerve resection (BCNR), simulating cavernosal nerve damage after radical prostatectomy, that this neuropraxia caused the early loss of corporal SMC, which the initial cell replication response could not counteract, and this was followed by fibrosis, leading ultimately to CVOD. The time course of iNOS, eNOS and nNOS expression supports the important role of iNOS.

iNOS

eNOS

nNOS

(J Sex Med 2009;6:415–428)
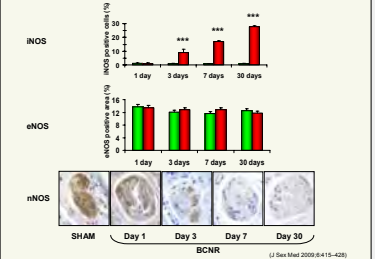
- Mimicking the antifibrotic effects of the endogenous tissue increase of iNOS, NO and cGMP cannot be achieved by the current clinical administration of PDE5i "on demand", where the peaks of corporal NO and/or cGMP levels are limited by the pharmacokinetics of each drug, and the duration of sexual stimulation. The current modality of PDE5i administration is merely palliative and not curative of the corporal histopathology that underlies CVOD.
- The long-term sustained administration of PDE5i, namely vardenafil (2.5 mg/kg/day; approx. equivalent to 25 mg/day in men, sildenafil (20 mg/kg/day; approx. equivalent to 200 mg/day in men), and tadalafil (5 mg/kg/day; approx. equivalent to 50 mg/day in men), to rats prevented the CVOD induced by both BCNR and unilateral CNR (UCNR)
- Drugs prevented the decrease in the penile corporal smooth muscle cell/collagen ratio, and the increase in apoptosis and reduction in cell proliferation, and partially counteracted the increase in collagen
- The long-term inhibition of iNOS activity exacerbated corporal fibrosis and CVOD in the BCNR rats, but sildenafil functional effects were not affected by L-NIL.

(BJU 2008;101: 1156-64)

- We also demonstrated, that implantation of muscle derived stem cells (MDSC) into the corpora cavernosa corrected the impaired erectile response induced by electric field stimulation of the cavernosal nerve (EFS) in aged rats.

OBJECTIVES

We aimed to investigate in the BCNR r at model whether CVOD, and the underlying loss of smooth muscle and fibrosis in the corpora cavernosa, caused by cavernosal nerve damage subsequent to radical prostatectomy may be prevented by:

a) a long-term continuous administration of sildenafil as opposed t o current on demand regimens, at lower doses t han the ones previously tested in this model, of a nitric oxide donor (molsidomine), or of both agents combined; and

b) corporal implantation of skeletal muscle derived stem cells (MDSC) alone or combined with oral administration of sildenafil at even lower doses

METHODS

Surgery and treatments: Nerve resection was performed by resecting the main cavernosal nerves by removing a 3-mm segment bilaterally (BCNR). In the sham-operated group, both cavernosal nerves were identified but not resected. Actual drug doses are expressed as mg/kg/day, with the approximate equivalent dose in men as mg/day (bold).

Animals: Five-month-old male Fisher 344 rats were treated and divided as follows:
Sildenafil at lower doses, with or without NO donors (n=8/group): 1) Sham; 2) BCNR untreated; 3) BCNR, sildenafil low dose, in water: 2.5 (**25**); 4) BCNR, sildenafil medium dose, in water: 10 (**100**);5) BCNR, sildenafil low dose, retrolingual: 2.5 (**25**); 6) BCNR, molsidomine IP: 10 (**100**), 7) BCNR, molsidomine IP 10 (**100**), with sildenafil medium dose in water 10 (**100**); and 8) BCNR, molsidomine IP 10 (**100**), with sildenafil low dose 2.5 (**25**).
Sildenafil at very low dose with or without MDSC implantation (n=8/group): 9) sildenafil, very low dose, in water: 1.25 (**12.5**); 10) sildenafil, very low dose in water: 1.25 (**12.5**) + MDSC (10⁶ cells), intracorporeal; 11) MDSC (10⁶ cells) alone, intracorporeal

Outcome methods: Dynamic Infusion Cavernosometry (DIC) was applied for measuring CVOD. Quantitative Masson trichrome and immunohistochemical staining in corporal tissue sections for alpha smooth muscle actin (αSMA) proliferating cell nuclear antigen (PCNA), and apoptosis were applied, confirming by quantitative western blots. Collagen was estimated by spectrophotometric picrosirius red elution method. The nNOS/neurofilament 70 dual fluorescence estimations for nerve regeneration are ongoing.

Statistical Analysis: Values were expressed as mean (M) ± standard error (SEM). Multiple comparisons among the different groups were analyzed by one-way ANOVA with Tukey post test. Differences among groups were considered significant at P<0.05.
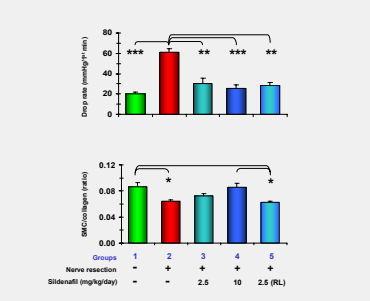
Figure 1

Effects of continuous long term treatment with sildenafil at doses lower than the ones previously used and different administration methods.
Top: All treatments normalized the high drop rate in the dynamic infusion cavernosometry and improved the response to intracorporeal papaverine (not shown).
Bottom: Quantitative image analysis. The SMC/collagen ratio was significantly increased but not completely normalized by treatment in all series, except group 5 (low dose, daily retrolingual administration). *P<0.05; **P<0.01; ***P < 0.005.

RESULTS

Effects of continuous long term treatment with sildenafil at lower doses, molsidomine with or without sildenafil, and MDSC with or without sildenafil at very low dose

✓ All treatments normalized the high drop rate in the dynamic infusion and improved the response to intracorporeal papaverine.
✓ The SMC/collagen ratio was significantly increased but not completely normalized by treatment in all series, and collagen content was reduced by sildenafil
✓ PCNA was unexpectedly reduced in series A and B.
✓ MDSC alone significantly increased the SMC/collagen ratio, but the combination with sildenafil at very low dose abrogated this effect.
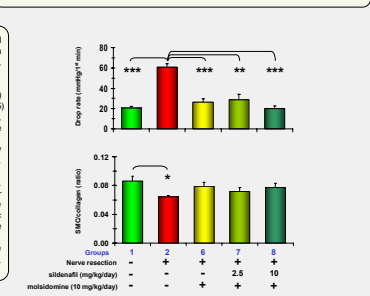
Figure 2

Effects of continuous long term treatment with NO donor molsidomine alone or in combination with sildenafil at lower doses
Top: All treatments normalized the high drop rate in the d ynamic infusion cavernosometry and improved the response to intracorporeal papaverine (not shown).
Bottom: Quantitative image analysis. The SMC/collagen ratio was increased but not completely normalized by treatment in all series. *P<0.05; **P<0.01; ***P < 0.005.
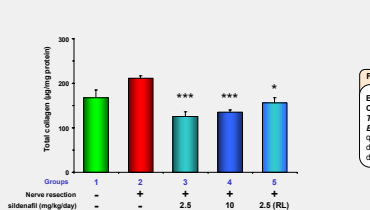
Figure 3

Effects of continuous long term treatment with NO donor molsidomine alone or in combination with sildenafil on total penile collagen content
The total collagen content was estimated from penile shaft homogenates by spectrophotometric picrosirius red elution method. All treatment significantly reduced the collagen content. *P<0.05, **P<0.005
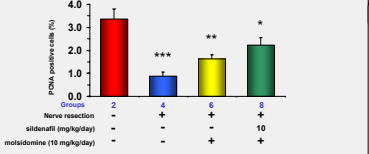
Figure 4

Effects of continuous long term treatment with NO donor molsidomine alone or in combination with sildenafil on PCNA
Quantitative image analysis. All treatment significantly reduced the percent of the PCNA positive cells assessed by immunohistochemistry. *P<0.05; **P<0.01; ***P < 0.005.
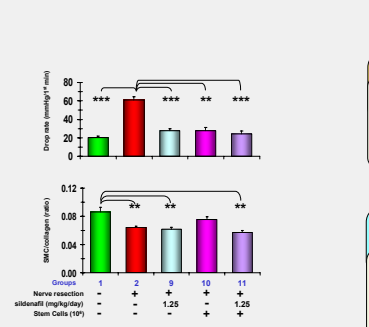
Figure 5

Effects of long-term sustained oral sildenafil treatment in very low dose on BCNR induced CVOD and smooth muscle loss/fibrosis with and without intracorporal MDSC implantation.
Top: Drop rate of intr acavernosal pressure measured by dynamic infusion cavernosometry.
Bottom: SMC/collagen ratio changes assessed by Masson trichrome staining followed by quantitative image analysis. All treatment are normalized the high drop rate, however the very low dose (1.5 mg/kg/day) sildenafil alone and in combination with MDSC was not able to prevent the decrease of the SMC/collagen ratio. **P<0.01; ***P < 0.005.

CONCLUSIONS

- An early long-term continuous treatment of the BCNR r at model wit h sildenafil at low daily doses prevent CVOD through correction of the underlying corporal histopathology, nearly as effectively as the higher doses previously tested.
- From the current results and our previous ones with tadalafil and vardenafil at relatively higher doses, it may be speculated that continuous long-term treatment with PDE5i at doses lower than the ones required to facilitate penile erection, as opposed to current on demand regimens, may be sufficient to prevent CVOD after radical prostatectomy.
- We also propose that the intracorporeal implantation of muscle derived stem cells may facilitate corporal tissue repair and prevent CVOD subsequent to radical prostatectomy.
- These results provide scientific bases for the emerging clinical interest in preventing or "curing" ED with PDE5i, rather than merely using them palliatively to facilitate an erection, and for pharmacological "penile rehabilitation" after RP.
- Further studies are needed to optimize the cell type, regimen of implantation, and the combination or not with PDE5i

CLINICAL FOLLOW UP

- Tadalafil is approved for daily use and both 5 and 2.5 mg tablets appear to be effective in a 14 d ay treatment (Seftel et al, J Uro l 2011, 185:243-48), although longer studies are needed to establish curative effects
- Nightly sildenafil (50 mg) for 9 months one month after RP enhance penile recovery (McCullough AR, J Urol 2010, 183:2451-6)

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- *Vardenafil prevents fibrosis and loss of corporal smooth muscle that occurs after bilateral cavernosal nerve resection in the rat.* Ferrini MG, Davila HH, Kovanecz I, Sanchez SP, Gonzalez-Cadavid NF, Rajfer J. Urology. 2006 Aug;68(2):429-35.
- *Long-term continuous treatment with sildenafil ameliorates aging-related erectile dysfunction and the underlying corporal fibrosis in the rat.* Ferrini MG, Kovanecz I, Sanchez S, Vernet D, Davila HH, Rajfer J, Gonzalez-Cadavid NF. Biol Reprod. 2007 May;76(5):915-23.

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- *Stimulating vaginal repair in rats through skeletal muscle-derived stem cells seeded on small intestinal submucosal scaffolds.* Ho MH, Heydarkhan S, Vernet D, Kovanecz I, Ferrini MG, Bhatia NN, Gonzalez-Cadavid NF. Obstet Gynecol. 2009 Aug;114(2 Pt 1):300-9.
- *Effect of muscle-derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat.* Nolazco G, Kovanecz I, Vernet D, Gelfand RA, Tsao J, Ferrini MG, Magee T, Rajfer J, Gonzalez-Cadavid NF. BJU Int. 2008 May;101(9):1156-64.

ACKNOWLEDGEMENT

CURRICULUM VITAE

A.   PERSONAL INFORMATION

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SOCIAL SECURITY:

FAMILY:

FOREIGN LANGUAGES: Spanish (native); French (reading,writing)

B.   EDUCATION AND DEGREES

Graduation Date	Degree	Subject	Institution	Country
12/61	M.Sc.	Biochemistry*	University of Buenos Aires	Argentina
03/64	Ph.D.	Biochemistry	University of Buenos Aires	Argentina
10/67	Ph.D	Biochemistry	University of London	England

* License for clinical chemistry and other biochemistry professional practice in Argentina.

C.   FELLOWSHIPS AND HONORS

1961	Gold Medal to the top graduate, University of Buenos Aires
1961-63	Research Fellowship, National Council of Scientific Research (Argentina)
1964	Gold Medal to the best Ph.D. thesis, University of Buenos Aires
1964-66	Research Fellowship (abroad), National Council of Scientific Research Argentina, to work at the Courtauld Institute of Biochemistry, London University, London, England
1967	Research Fellowship, Welcome Trust (England), to work at the same institution
1980	Senior Research Fellowship, Guggenheim Foundation (USA), to work at the California Institute of Technology, Biology Division, Pasadena, California, USA
1984	Short Training Fellowship, International Union Against Cancer, to work at the City of Hope Medical Center, Division of Biology, Duarte, California, USA
1987	Eleanor Roosevelt Senior Research Fellowship, International Union Against Cancer, to work at UCLA Medical School, Department of Medicine, Los Angeles, California, USA
1988	Senior Research Fellowship, United Nations University, to work at the same institution
1991	Harbor-UCLA, Research and Education Institute, Award to scientific research by institutional PhDs
1990-92	Public Health Award NIH-NIDDK 5F33 DK 08514-02, to work at the Population Research Center, Torrance, Ca
1993-2008	See various awards under pertinent sections below, particularly H, J, K
2009-life	Invited member, IberoAmerican Academy of Medical Sexology (Chair #23)
2009-13	Member, NIH UKGD Urology Study Section
2010-15	Editor-in-Chief, International Journal of Impotence Research, Nature Publishing Corp.

D. UNIVERSITY POSITIONS

1962/63	Research Associate, Institute of Histology and Embriology, Biochemistry Section, School of Medicine, University of Buenos Aires, Argentina
1963/64	Assistant Professor, Department of Organic Chemistry and Biochemistry, School of Biochemistry, University of Buenos Aires, Argentina
1968/69	Associate Professor, Department of Biochemistry, School of Science, Central University, Caracas, Venezuela
1969/92	Full Professor, Department of Cell Biology, School of Science, Central University, Caracas, Venezuela. Director, Laboratory of Molecular Oncology. On leave: 1990-92.
1990-5	Adjunct Associate Professor of Urology, Department of Urology, UCLA School of Medicine, Los Angeles, CA, USA. Joint with appointment at Harbor-UCLA Research and Education Institute.
1993-on	Director, Urology Research Laboratory, Harbor-UCLA Research and Education Institute, Torrance, CA
1995-on	Adjunct Professor of Urology, Department of Urology, UCLA School of Medicine, Los Angeles, CA, USA. Currently, Step IV
1997-on	Professor of Medicine, Department of Internal Medicine, Division of Endocrinology, Metabolism and Molecular Medicine, Charles R. Drew University of Medicine and Science, Los Angeles, Ca. Currently Step VI
2001-2006	Director, NIH-RCMI DNA Repository and Molecular Medicine Core, Charles R. Drew University of Medicine and Science, Los Angeles, Ca. Resubmitted as NIH-RCMI Molecular Medicine Research Core, sub-contracting LABioMed at Harbor-UCLA.
2004-2009	Associate-Director, NIH Program for the Biological Effects of Androgens in Men and Women. Charles R. Drew University/UCLA. Charles R. Drew University of Medicine and Science, Los Angeles, Ca

E. VISITING FACULTY POSITIONS

May 1971	Visiting Scientist, Massachusetts Institute of Technology, Division of Biology, Cambridge, Massachusetts, USA
Nov 1978/ Dec 1979	Gosney Visiting Associate in Biology, California Institute of Technology, Biology Division, Pasadena, California, USA
Oct 1980/ Sept 1981	Visiting Associate in Biology, same institution
Jul-Aug1982	Visiting Professor, University of Buenos Aires, Argentina, School of Biochemistry, Buenos Aires, Argentina
Jan 1987/ 1988	Visiting Professor, UCLA School of Medicine, Division of Hematology/ July Oncology, Los Angeles, CA, USA
Aug. 1988/ March 1989	Visiting Scientist, UCLA School of Medicine, Division of Urology, Los Angeles, California, USA

F.   POSTGRADUATE CERTIFICATES

1963	"Use and handling of radioisotopes". License by the National Authority of Atomic Energy, Argentina, two month full-time course.
1972	"Cancer Chemotherapy". 10h Course in the IV Latin American Course of Pharmacology, Caracas, Venezuela.
1969/76	Several independent 15-30h courses given by scientists from U.S. and European universities, in Caracas, Venezuela on different aspects of cell and molecular biology, (Campbell, Mahler, Attardi, Sabatini, Cordes, etc.)
1974	"Molecular bases of Pharmacology", L. Aronow (Stanford), 60 h course given in Caracas, Venezuela.
1975	"Methods for in the in vitro detection of carcinogens", United Nations 80 h Course, Mexico City, Mexico.
1978	"Stem-cell cloning in cancer research". 15h course, University of Texas, Tucson, Texas.
1982	"Basic Oncology". 30-h International course on the molecular and cell biology bases of cancer (Gallo, Riggs, Fidler, Klein, Anders, Magee, Klavins, etc.). Universidad Central, Caracas, Venezuela.
1985	"Chromosome alterations in cancer". J. Yunis, (Boston University), 12 h course Hospital Vargas, Caracas, Venezuela.
1987	"DNA probes in the practice of medicine", 20 h course, American Medical Association, Los Angeles, California, USA.
1988	"Biotechnology", Diploma from United Nations University for research work carried out at UCLA.

G.   PROFESSIONAL MEMBERSHIP

1965-9	Biochemical Society, Great Britain.
1968-90	Venezuelan Biochemical Society, Venezuela.
1968-90	Venezuelan Association for the Advancement of Science, Venezuela.
1981-	American Association for the Advancement of Science, USA.
1987-9	Tissue Culture Association, USA.
1989	New York Academy of Sciences, USA..
1992-on	American Society of Andrology, USA.
1998-on.	Endocrine Society, USA
2000-on	Society of Impotence Research (Current: Sexual Medicine Society of North America)
2001-on	American Urological Association
2001-on	Society for the Study of Reproduction
2004-on	Sexual Medicine Society of North America

H.   CONSULTING ACTIVITIES

Ad-hoc Reviewer:

NIH: NIDDK, NIH (1995). Center Program Grants on Prostate Growth NIDDK;
NIH (2001) Diabetes Research and Training Center; Welcome Trust, England (2002);
National Fund of Science and technology (FONCYT),
National Council for Scientific Research, Argentina (2002-2003);
Department of Defense Peer Reviewed Medical Research Program (PRMRP) (2004),
NIH Study Section (2009), in four different panels, including the Challenge grants

Consultant:

Merck Co (2001); Ligand Corporation (2002); Glaxo Smith Cline Laboratories (2003); Milkhaus Laboratory, Inc.(2003), Lily (2009)

I. PRESENTATIONS TO SCIENTIFIC MEETINGS

US, international only. Most as invited speaker, podium presentation, or Chair/Moderator (*). Others as poster contributor.

Aug. 1964	*5th Congress of Latin American Association of Physiological Sciences, Caracas, Venezuela.
Aug. 1965	*2nd Meeting of the Federation of European Biochemical Societies, Vienna, Austria.
Aug. 1967	*7th International Congress of Biochemistry, Tokyo, Japan.
Dec. 1968	*International Symposium of Nuclear Physiology and Differentiation, Belo Horizonte, Brazil.
Sept. 1970	*8th International Congress of Biochemistry, Montreux, Switzerland.
July 1971	*10th Congress of the Latin American Association of Physiological Sciences, Caracas, Venezuela.
July 1971	*1st Congress of the Pan American Association of Biochemical Societies, Caracas, Venezuela.
Nov. 1971	*11th Latin American Symposium "Protein and Nucleic Acid Synthesis, La Plata, Argentina. Symp. Speaker.
July 1973	*9th International Congress of Biochemistry, Stockholm, Sweden.
Oct. 1974	*11th International Cancer Congress, Florence, Italy.
Oct. 1974	*Symposium on "Molecular Base of Malignancy," Vienna, Austria.
July 1975	*Pre-FEBS Symposium No. 1: "Structure, synthesis and functions of nucleic acids in organelles", Paris, France.
July 1975	*10th FEBS Congress, Paris, France.
Aug. 1976	*2nd International Meeting on Genetics and Bio-genesis of Chloroplasts and Mitochondria, Munich, W. Germany.
July 1976	*10th International Congress of Biochemistry, Hamburg, W. Germany.
Sept. 1978	*2nd Congress of the Pan American Association of Biochemical Societies, Caracas, Venezuela.
Oct. 1978	*12th International Cancer Congress, Buenos Aires, Argentina.

April 1980	*VII Meeting of the Brazilian Biochemical Society, Caxambu, Brazil. Plenary Lecturer.
April 1981	Batelle Conference on Genetic Engineering, Frederick, MA, USA.
Aug. 1981	*III PAABS Congress, Mexico City, Mexico.
Feb. 1982	2nd Annl Congress for recombinant DNA research, Los Angeles, CA, USA.
Oct. 1984	*IV PAABS Congress, Buenos Aires, Argentina..
April 1986	UCLA Symposium on Oncogenes and Growth Factors, Steamboat Springs, Colorado, USA.
March 1987	Chemical Frontiers in Biotechnology, California Institute of Technology, Pasadena, California, USA.
Oct. 1987	*Cancer and aging, St. Louis, Missouri, USA.
Nov. 1987	DNA Probes in the Practice of Medicine, American Medical Association, Los Angeles, California, USA.
March 1990	Biological effects of androgens, Tampa, Fl
April 1991	UC Riverside/Nichols Institute Symposium on Cellular and Molecular Endocrinology, Riverside, CA
May 1991	*American Society of Andrology, 15th Annual Meeting, Montreal, Canada
Febr. 1992	*American Federation for Clinical Research, Western Regional Meeting, Carmel, CA
May 1992	*American Society of Andrology, 16th Annual Meeting, Bethesda, Md
June 1992	Nitric Oxide. Implications for Drug Research, Philadelphia, Pa
Febr. 1993	*American Federation for Clinical Research, Western Regional Meeting, Carmel, CA
May 1993	*American Society of Andrology, 18th Annual Meeting, Tampa, Fa
July 1993	*Endocrine Society, 75th Ann. Meeting, Las Vegas, Nv
March 1994	*IBC Third Symposium on Nitric Oxide, Philadelphia, PA
April 1994	*AFAR Meeting, Harriman, NY, USA
July 1994	Biology of Nitric Oxide, Los Angeles, CA, USA
May 1995	American Urological Association, Las Vegas, Nv, USA
June 1995	*76th Endocrine Society Meeting, Washington DC, USA
Sept 1995	Internatl Meeting of Nitric Oxide, Amelia Island, FA
April 1996	*Andrology Meeting, Minneapolis, WI
May 1996	*American Urological Association Meeting, Orlando, FL
June 1996	Endocrine Society Meeting, San Francisco, CA
July 1996	Nitric Oxide Meeting, Los Angeles, CA
Nov 1996	*Internatl Congress of Impotence, San Francisco, CA
May 1997	American Urological Association, New Orleans, LA
Dec 1997	IBC Meeting on Gene Therapy, San Diego, CA
March 1998	American Society of Andrology , Long Beach, CA
June 1998	*Endocrine Society, New Orleans, LA
Nov 1998	*NMHCC Biotechnology Conference, Washington, D.C.
March 1999	*UNESCO Advanced Course of Molecular Biology, Caracas, Venezuela
May 1999	American Urological Association Meeting, Dallas, TX
June 1999	*WHO Symposium on Erectile Dysfunction, Paris France
July 1999	*Endocrine Society, San Diego, CA
July 1999	*Prous TeleSymposium, Barcelona, Spain

May	2000	*American Urological Association Meeting, Atlanta, GA
July	2000	*Endocrine Society, Toronto, Canada
Sept	2000	*Society of Impotence Research, Cleveland, OH
June	2001	*American Urological Association Meeting, Anaheim, CA
July	2001	*Endocrine Society, Denver, CA
Dec	2001	*Society of Sexual Medicine, Charleston, NC
May	2002	American Urological Association Meeting, Orlando, Fl
July	2002	Endocrine Society, San Francisco, CA
Aug.	2002	Society for the Study of Reproduction, Baltimore, MD
Nov	2002	*Latinoamerican Association of Sexology, Margarita Island, Venezuela
Dec	2002	RCMI Meeting, Honolulu, HI
March 2003		*World Congress of Sexology, La Habana, Cuba
April	2003	*American Urological Association Meeting, Chicago, IL
June	2003	*Endocrine Society, Philadelphia, PA
July	2003	*Second WHO Symposium on Erectile Dysfunction, Paris France
Dec	2003	*Teleconference 2. American Confederation of Urology (In Spanish, to Latin American Affiliates), organized from Buenos Aires, Argentina
June	2004	*Seventh International Symposium on Neurobiology and Neuroendocrinology of Aging", Bregenz, Austria
Oct 2004		*Eleventh World Congress of the International Society for Sexual and Impotence Research. ISSIR 2004, Buenos Aires, Argentina
May 2005		*American Urological Association Meeting, San Antonio, TX
June 2005		*Endocrine Society, San Diego, CA
Nov 2005		Meet Sex Medic Soc North Am, New York (NY)
May 2006		American Urological Association Meeting, Atlanta, GA
June 2006		Endocrine Society, New Orleans, MI
Nov 2006		*Meet Sex Medic Soc North Am, Las Vega, NV
Dec 2006		*RCMI Meeting, San Juan, Puerto Rico
Nov 2007		Meet Sex Medic Soc North Am,
May 2007		American Urological Association Meeting,
June 2007		Endocrine Society
May 2008		American Urological Association Meeting, Orlando, FL
Aug 2009		*AUA Course, Baltimore, MD
Nov 2008		*Meet Sex Medic Soc North Am, Toronto, Canada,
March 2009		*NIH Meeting Urological Complications of Diabetes, Baltimore, MD
May 2009		*American Urological Association Meeting, Chicago Il
June 2009		Endocrine Society Meeting, Washington DC, MD
July 2009		*III International Consultation on Sexual Dysfunction, Paris France
Sept 2009		*Military Health Research Forum 2009, Kansas City
Nov 2009		*Meet Sex Medic Soc North Am, San Diego, CA
April 2010		American Urological Association Meeting, Annaheim, CA
Oct 2010		*XV Congreso Latino Americano de Sexualidad y Educación Sexual, Alicante Spain
Nov 2010		*Eur Soc Sexual Medic, Malaga, Spain
Nov 2010		Meet Sex Medic Soc North Am, San Diego, CA
March 2011		Impact: Innov Minds in Prost Res, CDMRPCures Meet, Orlando, FL

April 2011   102 Ann meet Am Assoc for Cancer Research (AACR), Orlando, FL
May 2011   American Urological Association Meeting, Chicago, CA

Articles and interviews with media

2002: El Universal (Caracas, Venezuela),
2002: Playboy (USA);
2003,2005: Noticias (Buenos Aires, Argentina)

I.   <u>**TEACHING EXPERIENCE**</u>

a.   1963-4- Instructor, teaching to undergraduate general biology students.
      1. Pathological biochemistry (10h, within 75h course per term).
      2. Biochemistry laboratory techniques (supervision: 20h/term).

b.   1969-1989 (with interruptions) Teaching to undergraduate general biology students.
      1. General biochemistry (35h, within 75h course per term).
      2. Biochemistry laboratory techniques (supervision: 40h/term).

c.   1971-1989 (with interruptions). Teaching to undergraduate biochemistry or cell biology students and/or to M.D.s and biochemists in post-graduate studies (Ph.D. in Cell Biology, and others).

      3. Instrumentation in biochemistry (12h, within 75h/course).
      4. Pathological biochemistry (20h, within 70h course).
      5. Special techniques in biochemistry (research lab work: 90h).
      6. Molecular bases of carcinogenesis and cancer chemotherapy (20h).
      7. Mitochondrial structure, function and biogenesis (8h).
      8. Oncogenes and growth factors (shorter version of No. 6, 12 h).
      9. Control of protein and nucleic acid synthesis (10h)
      10. Steroid hormone receptors and gene expression (8h)
      11. Role of nitric oxide in the control of smooth muscle tone and growth (6 h)
       Also some other minor topics in different courses, seminars, etc.

c.   Thesis/research training supervisor (not involving regular course teaching).

      1. At the undergraduate level: 40 students
      2. For M.Sc. thesis: 33 graduate students
      3. For Ph.D. thesis: 7 graduate students
      4. At the post-doctorate level: 25 trainees (Ph.D.s, MDs, Medical residents, VDs.)

d.   Participation in NIH/UCLA training programs

      1. Reproductive endocrinology, Harbor-UCLA Medical Center
      2. Prostate cancer, UCLA

3. Urology resident laboratory training, UCLA/Harbor-UCLA
4. 1995-1998. Journal Club and lab seminars, Harbor-UCLA Medical Center
5. 1997-present. Journal Club and lab seminars, Charles R. Drew University
6. 2000-present RCMI Molecular medicine Core, Charles R. Drew University

e. Pre- and Postdoctoral Trainees in 1996-2009

Previous trainees are too numerous to list. For the most recent see Table below. All trainees below spent one year or more of virtually full time research under NGC supervision

Faculty Member Past and Current Trainees	Training Period	Prior Academic Degree			Title of Research Project	Current Position (past trainees) Source of Support (current trainees)
		Degree	Year	Institution		
Past Trainees Penson D (UCLA Resident)	94-95	MD	91	David Geffen School of Medicine at UCLA	Hormonal control of erectile function	Assistant Professor, Department of Urology, Keck School of Medicine, USC, Los Angeles, CA
Garban H (Research fellow)	95-96	MD	92	Central U of Venezuela	Cloning of nitric oxide synthases and gene therapy of erectile dysfunction	Assistant Professor UCLA School of Medicine, Los Angeles, CA
Gelman J (UCLA Resident)	95-96	MD	92	David Geffen School of Medicine at UCLA	TGF beta expression in corpora cavernosa during aging	Assistant Professor, UC Irvine, CA
Moody J (UCLA Resident)	95-96	MD	92	David Geffen School of Medicine at UCLA	Gene therapy of erectile dysfunction in aging	Urology practice
Ryndin I (Pre-residency research training)	96	MD	91	U of Kiev, USSR	Expression and role of NMDA receptor in	Urology practice, New York

					urogenital tissues	
Bonavera JJ (Endocrinology fellow)	96-97	PhD	81	U of Buenos Aires, Argentina	Inducible nitric oxide synthase in the hypothalamus in aging	Private Veterinarian Practice
Xie Y (Minority fellow)	96-97	MD	90	Tongji Medical U, China	Effects of smoking on erectile function	Clinical studies, NIH SRA, Bethesda, MD
Byflower, F (CalState, Domingue Hills MS student)	98-01	BS			Myostatin regulation of myoblast proliferation	MS pursuing PhD studies in New York
Sheik, K (CalState MS student, as above)	98-01	BS			Regulation of myostatin promoter activation	MS, MBA, in laboratory supplies industry
Artaza J (PhD student BsAs, then NIH MBRS PI)	99-06	MS PhD			Myostatin effects on myoblast differentiation	Assistant Professor, CDU
Ferrini, M (Trainee in erectile dysfunction research)	99-01	PhD	95	U of Buenos Aires, Argentina	Role of nitric oxide in aging-related erectile dysfunction;	Assistant Professor UCLA School of Medicine, Los Angeles, CA / Assistant Professor CDU
Smith, N (CalState L.A., MS student)	00-01	BSc	99	Cal State, Los Angeles, CA	Expression of nitric oxide synthases in aging	Private Company, Oakland, CA
Sun J (GCRC medical student research)	00-01	MD	00	David Geffen School of Medicine at UCLA	DNA microarrays in Peyronie's disease and Role of PIN in erectile dysfunction	Family Medicine Practice
Valente, E	01-03	BSc	00	California State	Effect of long-	Scientist,

(CalState Dominguez Hills, MS student)				Dominguez Hills, Carson, CA	term PDE5 inhibitors on fibrosis in Peyronie's disease	AMGEN, Thousand Oaks, CA
Almeida FG (foreign fellow)	02-04	MD	00	U of Sao Paulo, Brazil	Androgen receptors in the vagina	Assistant Professor, U of Sao Paolo, Brazil
Porszasz Reisz S (NIH MBRS PI)	03-06	PhD			Myostatin overexpression in transgenic mice	Assistant Professor, CDU
Davila HH (pre-residency research training)	03-05	MD	02	Central U of Venezuela	Gene therapy of Peyronie's disease	Urology Resident, U of Florida, Tampa, Fl
Cantini, L (PhD student, Venezuelan Institute of Scientific Research)	04-06	MSc	03	U of Zulia, Maracaibo, Venezuela	Construction and use of adenoviral preparations for stem cell ex vivo engineering	PhD, Research Associate, University of Florida, Tampa, FA
Nolazco, G (NIH minority student)	04-06	MSc	04	California State Fullerton, CA	Molecular and cellular basis of urogenital fibrosis	Res Assoc, Charles R Drew U, Los Angeles, CA
Ng, C (medical student, pre-residency)	05-06	BSc-MD		UC Riverside, CA	Hormonal control of penile erection	Assistant Profesor, Urologist, Cedar's Sinai Hospital, Los Angeles, CA
Sanchez, S (NIH minority fellow)	05-07	MSc	02	California State Fullerton, CA	Molecular basis of corpora cavernosa and arterial fibrosis	Fellow, Harbor-UCLA, Torrance, CA Supported by LA BioMed
Rhambhatla, A (medical student, pre-residency)	06	BSc-MD	03 07	UC Riverside, CA David Geffen School of Medicine at UCLA	Effects of cavernosal nerve damage on corporal atrophy	Urology Resident, as from 06/07, Wayne State U, Detroit, MI
Heyderkhan, S	07-on	MSc	07	U of Göteborg,	Stem cells for	Fellow,

(fellow)				Göteborg, Sweden	the treatment of stress urinary incontinence	Harbor-UCLA LABioMed, Torrance, CA Supported by LA Biomed
Wang, J (GCRC medical student)	08-on	BSc	06	UC Riverside	Muscle-derived stem cells for the treatment of myocardial infarction	Medical Student (3rd-4th year), UCLA School of Medicine NIH M01RR00425
Rivera, S (GCRC medical student)	08-on	MD	08	UCLA	Effects of nitric oxide/cGMP on fibrosis of the corpora cavernosa and erectile dysfunction	Fellow, Harbor-UCLA LABioMed, Torrance, CA Supported by Urology Division. GCRC application 09-08
Ho, M (NIH SC2 grant PI)	08-10	MD			Stem cells in stress urinary incontinence	Initially Assistant Professor, Currently Associate Professor, at CDU
Chow, S (CDU/Western Grant)	09-11	PharmD			Biomarkers for following up myocardial infarction	Assistant Professor at Western University
Segura, D (MS student)	11-13	BS	CalState Dom Hills		Bisphenol A effects on penile erection	Ongoing, As from 01/11
Zhang, Y (PhD student)	11-13	MD		Dept. of Urology, the first affiliated hospital of Sun Yat-sen University(Guangzhou, China).	Induced pluripotent stem cells for erectile dysfunction	To start from 07/11

High school students not included

J. ADMINISTRATIVE AND CONSULTANT EXPERIENCE

1970-71   Member of the organizing Committee of the 10th Congress of Latin American Society of Physiological Sciences (ALALC).

1970-71   Member of the Organizing Committee of the 1st Congress of Pan American Association of Biochemical Societies (PAABS).

1973-75   Chairman of the Committee on Scientific Research, School of Biology, Central University of Venezuela.

1978   Member and main writer/editor of the Committee that prepared the text of a bill on the scientific investigator career in Venezuela, Venezuelan National Council of Scientific Research, CONICIT.

1977-78   Chairman of the Organizing Committee of the 2nd PAABS Congress.

1976-78   Member of Executive Council of the Venezuela Biochemical Society.

1978-79   Secretary General of the Pan American Association of Biochemical Societies, PAABS.

1979-80   Chairman of the Biology Grants Committee, CONICIT.

1979-85   Member of the Symposium Committee of the International Union of Biochemistry.

1981   Advisor to CONICIT on genetic engineering applications to biotechnology (see List of Public.No. 33)

1981   Advisor to CONICIT on the cooperation agreements with the US National Academy of Sciences.

1982-84   Vice-Chairman of the Committee on Genetic Engineering, CONICIT.

1974-78   Coordinator of the Research Group on Molecular Biology, School of Biology, Central University of Venezuela.

1985-87   Member of Technical Council, Center of Cell Biology, Central University of Venezuela.

1985-86   Member of Post graduate Committee on Cell Biology, Central University of Venezuela.

1985-89   Member of National Committee on Genetic Engineering and Biotechnology, appointed by the President of Venezuela.

1985   Scientific Consultant, Lopapa Institute Project.'Institute of Molecular Oncology.' 200-page description of laboratory services specialized in cancer tests derived from new concepts of cell and molecular biology.

1985-86   Member of the Committee on Retirements and Pension Plans of the National Council of Universities, and that of the Central University of Venezuela.

1986   Organizer of the Colombian/Venezuelan Meeting on Binational Biotechnology Projects, Cucuta, Colombia.

1986.   CONICIT representative to the Biotechnology Committee of SELA (Economic System of Latin America).

1989   Scientific Advisor to the Director of UNESCO on US molecular biology/genetic engineering institutions.

1993-on   Recombinant DNA Committee, Research and Education Institute, Harbor-UCLA Center

1998/9   Panel member. First International Consultation on Erectile Dysfunction. World Health Organization/International Union Against Cancer. Amsterdam, Dallas, Paris

1997   Career investigator (not-activated), National Council of Scientific and Technological Research (CONICET), Buenos Aires, Argentina.

1998-on   Ad hoc evaluator, National Fund of Science and technology (FONCYT), National

	Agency of Scientific and Technologic Promotion, Buenos Aires, Argentina
2001-on.	Director, RCMI DNA Repository and Molecular Medicine Core, Charles C. Drew University, Los Angeles, CA
2001-on.	Ad hoc consultant/lecturer: Merck, AMGEN, and several small corporations.
2002-on.	Member of Search Committee for Research Director, Female Sexual Medicine Center,: UCLA School of Medicine, Department of Urology
2003	Panel member. Second International Consultation on Erectile Dysfunction. World Health Organization/International Union Against Cancer. Amsterdam, Dallas, Paris
2005.	Ad hoc member, Department of Defense CDMRP Study Group
2008-on	Appointments and Promotion Committee, Charles Drew University
2008-on	Scientific Advisory Committee, Faculty Senate, Charles Drew University
2009	NIH Special Emphasis Study Group: for members of other study groups
2009	Ad hoc member, NIH Study Group: for Challenge grants
2009-13	Regular member NIH UKGD Study Section
2009	Panel member. Third International Consultation on Erectile Dysfunction. World Health Organization/International Union Against Cancer. Amsterdam, Dallas, Paris
2009-on	Appointments and Promotions Committee, College of Medicine, Charles Drew University
2010-on	Appointments and Promotions Committee, Faculty-wide. Charles Drew University
2010-on	Conflicts of Interest Committee, Charles Drew University

K.   AD HOC REVIEWER/JOURNAL POSITIONS

1. Journals: Endocrinology, Nature Medicine, American Journal of Physiology, Biology of Reproduction, Journal of Andrology, Journal of Urology, Urology, Nitric Oxide, Cancer; Journal of Endocrinology, Experimental Gerontology, Asian Journal of Andrology; Journal of Sexual Medicine; Endocrinology; Circulation Research, many others
2. NIH: NIDDK, NIH (1995). Center Program Grants on Prostate Growth
3. NIDDK, NIH (2001) Diabetes Research and Training Center
3. Welcome Trust, England (2002)
4. Department of Defense (2004), Skeletal muscle biology and pathology
5. Singapore University (2006), Faculty appointments
6. Editorial Board member (2007-on), Journal of Sexual Medicine
7. Editor in Chief, International Journal of Impotence Research (2010-15)
8. Member, Editorial Board, Archivos Espanoles de Urologia (2011 on)

L.   LECTURES NOT INCLUDED IN COURSES

Too many to list here. Roughly, they are about 4-7 per year, mostly at the LA area, but also elsewhere in the USA and Latin America

M.   RECENT FUNDING IN THE USA

Previous funding overseas (1969-1990) is not listed.

All grants as Principal Investigator, unless indicated otherwise (**only total direct costs listed**). "Total" indicates for the whole project period.

a. 1991-95 (funded)

Tobacco Related Disease Research Program, University of California (TRDRP): $480,000
NIH (Senior Fellowship Award):
American Federation for Aging Research (AFAR):
American Diabetes Association:
Merck Co:
NIREC Corp (1):
Research and Education Institute (REI)
Vollmer Foundation:
Various (mentor for postdoc funding: NIH, AFAR/Hartford, TRDRP, Vollmer Found., etc.)

b. 1994-96 (funded)

NIREC Corp (2):

c. 1999-2003-completed (funded)

1. REI-009290- Eli and Edythe L. Broad Foundation (Scientific PI: Gonzalez-Cadavid N; Recipient and Co-PI: Rajfer J)   20% effort
  "Molecular Pathophysiology of Peyronie's Disease"
05/01/99-06/01/03

2. RO1 DK53069-01 NIH/DDK (PI: Gonzalez-Cadavid N)   20% effort
   "Erectile Dysfunction and Nitric Oxide Synthase in Aging"
   05/01/99-04/30/03

d. Recently completed (funded) Only direct costs

3. RO1 DK53069-01A4 NIH/DDK (PI: Gonzalez-Cadavid N)   17% effort
 "Erectile Dysfunction and Nitric Oxide Synthase in Aging (Renewal)"
 05/01/03-04/30/08:
        Administrative supplement 1
        Administrative supplement 2
Renewal submission: 07/01/08

4. G12RR030262 NIH (PI of Program: Francis CK/Baker RS; Core Director and PI: Gonzalez-Cadavid N)   10% effort
 "RCMI Core Infrastructure Development Grant: DNA Repository and Molecular Medicine Core"
 09/01/00-08/31/06. Currently funded by institutional bridge grant
Renewal submission: 01/31/08

5. U54 HD41748-01 NIH/NICHHD (PI of Program: Bhasin S; PI Pilot grant: Gonzalez-Cadavid N)   10% effort
 "Androgen Stimulation of Myogenic Stem Cell Differentiation"
 10/01/03-09/30/06

6. REI 11423-01 Bayer Corporation (Scientific PI: Gonzalez-Cadavid N; Recipient and Co-PI: Rajfer J)   4% effort
 "Novel Therapeutic Application of Vardenafil for the Treatment of Peyronie's Disease and other Fibrotic Diseases"
 11/01/03-010/31/04

7. REI 11527-01 Lilly-Icos Corporation (Scientific PI: Gonzalez-Cadavid N; Recipient and Co-PI: Rajfer J)   5% effort
  "Effect of Long-term Treatment with Tadalafil on Phosphodiesterase-5 (PDE5) Expression in Human Penile Cell Cultures"
12/01/03-11/30/04

8. REI 11425-01. UCLA Dept of Urology (Co-PIs: Gonzalez-Cadavid N, Berma J) 5% effort
 "Molecular and Cellular Targets for the Therapy of Female Sexual Arousal Disorder (FSAD)"
 07/01/03-06/30/04

9. REI 11548-01. Takeda Pharmaceuticals North America (PI: Gonzalez-Cadavid N)   7% effort
 "Antifibrotic and Vasculoprotective Effects of Pioglitazone on Aging- and Diabetes-Related Erectile Dysfunction
12/15/03-12/14/04$128,000

10..LABioMed..........Pfizer Corporation. (Sci PI: Gonzalez-Cadavid N; Co-PI: Rajfer J)
 2% effort
Effects of long-term therapy with sildenafil on the histological and functional alterations of the aged corporal tissue; implications for reversal of corporal venooclussive dysfunction (CVOD).
12/01/04-11/30/05

11. LABioMed ....   Pfizer Corporation (Sci PI: Gonzalez-Cadavid N; Co-PI: Rajfer J)
 3% effort
Role of sildenafil in preventing the histological and functional alterations in the cavernosal tissue following cavernosal nerve damage; implications for the treatment of impotence following radical prostatectomy.
02/01/05-11/30/05

12. LABioMed........ Lilly ICOS (Sci PI: Gonzalez-Cadavid N; Co-PI: Rajfer J)   3% effort
Effect of tadalafil in preserving smooth muscle function following cavernosal nerve injury
06/01/05-05/31/06

13. LABioMed 12190-01 (PI: Gonzalez-Cadavid N; Co-PI: Torday J)   3% effort
Enrichment and characterization of stem cells from human skeletal muscle for the therapy of congestive heart failure (CHP).

03/01/07-02/28/07

14. ………American Diabetes Association (PI: Gonzalez-Cadavid N)   10% effort
Erectile dysfunction and vascular fibrosis in diabetes.
08/01/05-07/31/08

15. Takeda North America, Inc (Gonzalez-Cadavid)   4% effort
Antifibrotic and Renoprotective Effects of Pioglitazone on Type 2 Diabetes Related
Tubulointerstitial Fibrosis
04/01/08 - 03/31/08

16. Harbor/UCLA Division of Urodynamics. Pilot grant (Bathia/Ho/Gonzalez-Cadavid) 4%
effort
Reversion of levator ani atrophy by muscle derived stem cells in a rat model of stress urinary
incontinence
03/01/07-04/01/08

17. PR064756 Department of Defense (PI: Gonzalez-Cadavid N)   10% effort
Pharmacological prevention and reversion of erectile dysfunction after radical prostatectomy, by
modulation of nitric oxide/cGMP pathways
 04/01/07-03/31/10

18. NIH R21DK070003-01A1 (PI: Gonzalez-Cadavid N)   15% effort
Cell-selective expression of fibrotic gene pathways
08/01/07-07/31/10

19. GCRC Medical Student Program (Gonzalez-Cadavid, PI, Wang J, student) NIH-
GCRC/Norris Foundation
3% effort
Nitric oxide/cGMP modulation of skeletal muscle stem cell differentiation in myocardial
infarction in the rat
12/01/07-11/30/09

20. GCRC Medical Student Program (Gonzalez-Cadavid, PI, Rivera S, student) NIH-GCRC
                                                                  3% effort
Effects of nitric oxide/cGMP modulators on fibrosis of the penile corpora cavernosa and
vasculogenic erectile dysfunction caused by cavernosal nerve damage in a rat model
12/01/08-11/30/09

e. Ongoing. Only direct costs

Funded, ongoing

1. PR064756 (PI: Gonzalez-Cadavid) Dept of Defense
Dates: 03/01/07-02/28/10 (no cost extension to 03/31/11)

Title: Pharmacological prevention and reversion of erectile dysfunction after radical prostatectomy, by modulation of nitric oxide/cGMP pathways

2. PC061300 (PI: Gonzalez-Cadavid) Department of Defense
Dates: 03/31/07-02/28/11(no cost extension to 03/12)
Title: Modulation of stem cell differentiation and myostatin as an approach to counteract fibrosis in dystrophic muscle regeneration after injury.

3. U54 CA14393-01 (PI pilot project: Gonzalez-Cadavid; Program Director: Vadgama). NIH/NCI
Dates: 01/01/10-08/31/12
Title: Potential oncogenic effects of alcohol on breast stem cells (previous: Mechanism of alcohol-associated breast cancer)

4. NIH R21ES019465-01 (PI: Gonzalez-Cadavid) NIH/NIEHS
Dates: 09/01/10-08/31/12
Title: Bisphenol A effects on the peripheral mechanisms of penile erection

5. 5-U54-RR026138-02 (PI of pilot project: Gonzalez-Cadavid; Program Director: Norris) NIH/NCRR. In collaboration with LABioMed at Harbor-UCLA
Dates: 03/01/11-02/28/12
Title:Therapy of diabetes-related critical limb ischemia with muscle derived stem cells and NO donors

f. Approved but funding eventually declined or not honored

1. Takeda USA Corporation (PI Gonzalez-Cadavid)   10% effort
Effects of alogliptin alone and in combination with pioglitazone on diabetic nephropathy in a rat model
03/01/10-10/31/11
Funding declined because of Alogliptin Program suspension

6. Endogenous Stem Cells Activators (ESAI Inc) (PI Gonzalez-Cadavid)   20% effort
Pre-clinical study on the pharmacological modulation of endogenous stem cells in the penile corpora cavernosa for the correction and prevention of aging-related erectile dysfunction by using the Kronos IV line of compounds
03/01/10-02/28/13 (contract signed)
Defaulted

g. Submitted, under review or consideration

1. NIH/NIEHS…………… RFA-ES-10-009 (PI Gonzalez-Cadavid)
Cellular-molecular signature and mechanism of BPA effects on penile erection in response to the funding opportunity RFA-ES-10-009 "Research Consortium for 2-year Bisphenol A Toxicity Study (U01)"
10/01/11-09/30/15

2. NIH 1R21DK089996-01 (PI: Gonzalez-Cadavid)   10% effort
Human iPS in erectile dysfunction after radical prostatectomy in rat models
10/01/11-09/30/13
Previous Score: 30

3. NIH R21ES019465-01 (PI: Gonzalez-Cadavid, student: Denesse Segura) NIH/NIEHS
Minority Student Supplement to Bisphenol A effects on the peripheral mechanisms of penile erection
Dates: 04/01/11-08/31/12

g. To resubmit

1. RO1 DK53069-08 (PI: Gonzalez-Cadavid)   NIH/NIDDK.   15% effort
Erectile Dysfunction and Nitric Oxide Synthase in Aging
05/01/03-04/30/08 Renewal: 06/01/10-05/31/15

2. R21 DK085411-01 (PI: Gonzalez-Cadavid)   NIH/NIDDK   10% effort
Nitric oxide and cGMP modulation of Oct-4 renal stem cells in diabetic nephropathy
09/01/09-08/31/11

3. R21 DK085413-01 (PI: Gonzalez-Cadavid)   NIH/NIDDK   10% effort
PPAR gamma modulation of Oct-4 renal stem cells in diabetic nephropathy
09/01/09-08/31/11

4. R21   (previous Challenge grant) (PI Gonzalez-Cadavid NF)   10% effort
Modulation of human iPS differentiation in diabetic nephropathy in rat models
09/01/09-08/31/12

5. Norris Foundation (Co-PIs: Gonzalez-Cadavid NF, White R)   10% effort
Adult Stem Cells in Cardiovascular Disease Laboratory
06/01/10-05/31/12

g. Not funded or resubmitted

1. NIH/NIAMS R01 ARO53875-01 (Gonzalez-Cadavid)   20% effort
Myostatin and Stem Cells in Fibroadipogenic Degeneration of Dystrophic Muscle
04/01/2007 - 03/31/2012

2. G12RR030262 NIH (PI of Program: Francis CK/Baker RS; Core Director and PI: Gonzalez-Cadavid N)   20% effort
 "RCMI Infrastructure Development Grant: Molecular Medicine Research DNA Core"
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3. MBRS Program NIH (PI: Artaza J; Mentor and Co-I: Gonzalez-Cadavid N)   4% effort
 "Myostatin Effects on Murine Stem Cell Myogenesis."
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4.   NIH (PI: Ferrini M; Co-investigator: Gonzalez-Cadavid)   10% effort
Effects of Nitric Oxide and cGMP in a Model of Cavernosal Nerve Damage
04/1/2007 - 03/31/2012

5. American Diabetes Association (Magee, Co-investigator: Gonzalez-Cadavid.)   4% effort
Mechanism of Anti-fibrotic Effects of cGMP on Vascular Complication of Diabetes
07/1/2007 - 06/30/2009

6.. Lilly-ICOS (PI: Gonzalez-Cadavid N)   4% effort
 Effects of tadalafil on vascular calcification and ossification
04/01/07-03/31/08

7.   SanBio Inc (PI: Gonzalez-Cadavid N)   4% effort
Notch-engineered bone marrow stem cells for the therapy of erectile dysfunction and stress urinary incontinence
06/01/07-05/31/08

8. Animal Models of Diabetes Complications Consortium (PI: Gonzalez-Cadavid)
A diabetes mouse model for studying endogenous/exogenous stem cell interaction
08/01/10-07/31/11

9. Animal Models of Diabetes Complication Consortium (Co-I: Gonzalez-Cadavid)
Modulation of Stem Cell Differentiation in Type 2 Diabetic Nephropathy
08/01/10-07/31/11

10. NIDDK R21 (Nicholas/Gonzalez-Cadavid, Co-PIs)
Effects of diabetes on stem cell cross talk in renal tissue repair
07/01/11-06/30/13

N. LIST OF PUBLICATIONS

Communications to scientific meetings are only added to the respective paper reference when its manuscript appears as submitted or in preparation.

Published Research Papers (peer reviewed).

For Pubmed search, please use Gonz alez-Cadavid N, or the last name only, and not NF, to

avoid missing 40 odd papers

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P.   COMMUNICATIONS TO CONGRESSES AND SCIENTIFIC MEETINGS

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C120. Quian A, Rajfer J, **Gonzalez-Cadavid NF** (2002) Detection of potential regulators of nitrergic neurotransmission of penile erection in the rat penis. Soc Study Reprod , Baltimore, MD

C121. Lee KK, Ferrini M, Sinha-Hikim A, Wang C, Swerdloff R**, Gonzalez-Cadavid NF** (2002) Aging-Related Expression of p53 in the Hypothalamus and Other Brain Regions which Regulate Reproductive Functions. Endocr Soc Meet, San Francisco, CA

C122. **Gonzalez-Cadavid NF** (2002) Molecular basis of of male sexual response. Latin Amer Assoc Sexol Meet, Margarita Island, Venezuela

C123**. Gonzalez-Cadavid NF** (2002) Gene therapy of erectile dysfunction. Latin Amer Assoc Sexol Meet, Margarita Island, Venezuela

C124. **Gonzalez-Cadavid NF** (2002) Molecular and cellular basis of female peripheral sexual dysfunction. Latin Amer Assoc Sexol Meet, Margarita Island, Venezuela

C125. Authors to be inserted, **Gonzalez-Cadavid NF** (2002). Title to be inserted. RCMI Meet, Honolulu, HI

C126. Authors to be inserted, **Gonzalez-Cadavid NF** (2002). Title to be inserted. RCMI Meet, Honolulu, HI

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C128. Qian A, Rajfer J, **Gonzalez-Cadavid NF** (2003) Interactions of neuronal nitric oxide synthase with potential regulatory proteins expressed in the rat penis. Am Urol Assoc Ann Meet, Chicago, IL. J Urol #

C129. Davila H, Ferrini M, Rajfer J, **Gonzalez-Cadavid NF** (2003) Fibrin induction of a Peyronie's-like plaque in the rat penile tunica albuginea. a new model for Peyronie's disease. Am Urol Assoc Ann Meet, Chicago, IL . J Urol #

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C134. **Gonzalez-Cadavid NF** (2003) Integrating approach to sexual dysfunction-Perspectives from molecular biology and gene therapy. World Congress of Sexology, La Habana, Cuba

C135. **Gonzalez-Cadavid NF**, Rajfer J (2003) Animal models of aging-, diabetes-, and smoking-related erectile dysfunction. World Health Organization Workshop on Erectile Dysfunction, Paris, France

C136. **Gonzalez-Cadavid NF** (2003) Molecular biology of penile phosphodiesterases. World Health Organization Workshop on Erectile Dysfunction, Paris, France

C137. Artaza JN, Bhasin S, Reisz-Porszasz S, **Gonzalez-Cadavid NF** (2003). Lower cardiac muscle mass in transgenic mice that hyperexpress myostatin suggests role of myostatin in cardiomyogenesis. Am Feder Med Res, Western Meeting, Carmel, CA. J Investig Medic #.

C138. Singh R, **Gonzalez-Cadavid NF**, Taylor WE, Artaza J, Bhasin S (2003) Inhibition of adipogenic differentiation in pluripotent C3H 10T1/2 stem cell line by androgens. Am Feder Med Res, Western Meeting, Carmel, CA. J Investig Medic #

C139. Reisz-Porszasz S, Bhasin S, Artaza JN, Shen R, Sinha-Hikim I, Hogue A, **Gonzalez-Cadavid NF** (2003) Decreased skeletal muscle mass in transgenic mice with muscle-specific hyperexpression of myostatin. Am Feder Med Res, Western Meeting, Carmel, CA. J Investig Medic #

C140. Artaza JN, Bhasin S , Reisz-Porszasz S, **Gonzalez Cadavid NF** (2003) Reduction in cardiac muscle mass in transgenic mice that hyperexpress myostatin suggests a role of myostatin in cardiomyogenesis. Endocr Soc Ann Meet, Philadelphia, PA. Selected from more than 2,500

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C141. Singh R, Artaza JN, **Gonzalez-Cadavid NF**, Taylor W, Datta A, Bhasin S (2003). Testosterone and dihydrotestosterone inhibit adipogenic differentiation of preadipocyte cell line 3T3-L1. Endocr Soc Ann Meet, Philadelphia, PA.

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C143. Singh R, Artaza JN, **Gonzalez-Cadavid NF**, Taylor WE, Datta A, Bhasin S (2003). Testosterone and dihydrotestosterone inhibit adipogenic differentiation of preadipocyte cell line 3T3-L1: Possible role of androgen receptor and Wnt signaling pathway. The Endocrine Society's Hot Topics in Endocrinology Symposium, San Diego. CA

C144. Artaza JN, Bhasin S, **Gonzalez-Cadavid NF** (2004) Myostatin inhibits myogenesis and promotes adipogenesis in 10T1/2 mesenchymal pluripotent cells. Am Feder Med Res, Western Meeting, Carmel, CA. J Investig Medic #

C145. Artaza JN, Bhasin S, Shen R, **Gonzalez-Cadavid NF** (2004) Myostatin inhibits myogenesis and promotes adipogenesis in 10T1/2 mesenchymal pluripotent cells. Am Feder Med Res, Western Meeting, Carmel, CA. J Investig Medic #. . Winner of the Glaxo-Smith-Kline Endocrinology Scholar Award.

C146. Singh R, Artaza JN, **Gonzalez-Cadavid NF**, Bhasin S (2004) Role of androgen receptor and Wnt signaling during testosterone and dihydrotestosterone-induced inhibition of adipogenesis in 3T3-L1 cells. Am Feder Med Res, Western Meeting, Carmel, CA. J Investig Medic #. Winner of the Glaxo-Smith-Kline Endocrinology Scholar Award

C147. Davila HH, Ferrini MG, Magee TR, Rajfer J, **Gonzalez-Cadavid NF** (2004) Detrimental effect of cigarette smoking on erectile dysfunction in the rat is contingent to diabetes as co-morbidity. Am Urol Assoc Ann Meeting, San Francisco, CA

C148. Davila HH, Magee TR, Rajfer J, **Gonzalez-Cadavid NF** (2004) Gene therapy with the inducible nitric oxide synthase (iNOS) cDNA regresses the fibrotic plaque in an animal model of Peyronie's disease**.** Am Urol Assoc Ann Meeting, San Francisco, CA

C149. Vernet D, Qian A, Magee TR, Ferrini MG, Rajfer J, **Gonzalez-Cadavid NF** (2004) Osteogenic progenitor cells in the Peyronie's disease plaque may be responsible for plaque ossification. Am Urol Assoc Ann Meeting, San Francisco, CA

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C151. Ferrini MG, Valente E, Rajfer J, **Gonzalez-Cadavid NF** (2004) Long-term treatment with high doses of sildenafil does not up-regulate the levels of Phosphodiesterase 5 (PDE5) in the rat penis. Am Urol Assoc Ann Meeting, San Francisco, CA

C152. Vernet D, Ferrini MG, Qian A, Rajfer J, **Gonzalez-Cadavid NF** Phosphodiesterase 4 (PDE4) is expressed in the normal tunica albuginea and in the Peyronie's disease plaque (2004). Am Urol Assoc Ann Meeting, San Francisco, CA

C153. Kovanecz I, Ferrini MG, Davila HH, Rajfer J, **Gonzalez-Cadavid NF**. (2005) Pioglitazone ameliorates penile corpora veno-occlusive dysfunction (CVOD) in the aged rat. Am Urol Assoc Ann Meeting, San Antonio, TX

C154. Davila HH, Miranda-Sousa AJ, Kovanecz I, Carrion RE, Miller C, Salup R, **Gonzalez-Cadavid NF**, Rajfer J. (2005) Effect of bilateral cavernosal nerve resection on the histological alterations in the penile vascular system. Am Urol Assoc Ann Meeting, San Antonio, TX

C155. Davila HH, Carrion RE, Salup R, Rajfer J, **Gonzalez-Cadavid NF** (2005) Alterations of the corpora cavernosa associated with aging and veno-oclussive dysfunction in the rat. Am Urol Assoc Ann Meeting, San Antonio, TX

C156. Davila HH, Magee TR, Zuniga F, Rajfer J, **Gonzalez-Cadavid NF**. (2005) Plasminogen activator inhibitor-1 (PAI-1) is increased in human Peyronie's disease. Am Urol Assoc Ann Meeting, San Antonio, TX

C157. Vernet D, Magee TR, Qian A, Nolazco G, Rajfer J**, González-Cadavid NF.** (2005) Tadalafil does not up-regulate the expression and activity of phosphodiesterase 5 (PDE-5) in the penis. Am Urol Assoc Ann Meeting, San Antonio, TX

C158. Rajan Singh, Jorge N. Artaza, Wayne Taylor, Melissa Braga, Shalender Bhasin **and Nestor F Gonzalez-Cadavid** (2005) Testosterone Induced Changes in the Expression Profile of Transforming Growth Factor-Beta Signaling Pathway Genes in Mesenchymal Pluripotent C3H 10T1/2 Cells. The Endocrine Society 87th Annual Meeting June 2005, San Diego, CA.

C159. R. Singh, WE. Taylor, JN. Artaza, M. Braga, **NF. Gonzalez-Cadavid**, S. Bhasin (2005) Testosterone modulation of myogenic and adipogenic differentiation in pluripotent cell line C3H/10T1/2 by androgen receptor-dependent regulation of Wnt/β-catenin pathway. The Endocrine Society 87th Annual Meeting June 2005, San Diego, CA

C160. Kovanecz I, Ferrini MG, Rajfer J, **Gonzalez-Cadavid NF**. Amelioration of aging related penile corporal veno-occlusive dysfunction (CVOD) in rats by pioglitazone. *Annual Meeting of SMSNA* 2005, New York, NY, USA

C161. Magee TR, Kovanecz I, Cantini L, Ferrini MG, Vernet D, Davila HH, Rajfer J, and **Gonzalez-Cadavid NF**. A short hairpin (sh)RNA targeting PIN (Protein inhibitor of NOS) is more efficient than the anti-sense PIN RNA for gene therapy of aging-related erectile

dysfunction in the rat. 2006 Sexual Medicine Society of North America Fall Annual Meeting.

C162. Magee TR, Kovanecz I, Cantini L, Ferrini MG, Vernet D, Davila HH, Rajfer J, and **Gonzalez-Cadavid NF**. A short hairpin (sh)RNA targeting PIN (Protein inhibitor of NOS) is more efficient than the anti-sense PIN RNA for gene therapy of aging-related erectile dysfunction in the rat. 2006 Research Centers in Minority Institutions Conference.

C163. Magee TR, Kovanecz I, Cantini L, Ferrini MG, Vernet D, Davila HH, Rajfer J, and **Gonzalez-Cadavid NF**. A short hairpin (sh)RNA targeting PIN (Protein inhibitor of NOS) is more efficient than the anti-sense PIN RNA for gene therapy of aging-related erectile dysfunction in the rat. 2007 American Urological Association Annual Meeting.

C164. Ferrini MG, Cantini LP, Vernet D, Magee TR, Qian A, Gelfand RA, Rajfer J, **Gonzalez-Cadavid NF**. Pro-fibrotic role of myostatin in Peyronie's disease. 2008 American Urological Association Annual Meeting.

C165. Androgen modulation of cell lineage commitment by muscle-derived stem cells (MDSC) J. Tsao, D. Vernet, G. Nolazco, JN Artaza, **NF Gonzalez-Cadavid**. Presented at the Western American Federation for Medical Research, Winner Travel Award, February, 1-4, 2006. Sunset Center Carmel, CA, USA.

C166. TR Magee, JN Artaza, MG Ferrini, D Vernet, F I. Zuniga, L Cantini, S Reisz-Porszasz, J Rajfer, **NF Gonzalez-Cadavid**. Myostatin short interfering hairpin RNA gene transfer increases muscle mass and reduces interstitial connective tissue. Presented at the Western American Federation for Medical Research, February, 1-4, 2006. Sunset Center Carmel, CA, USA.

C167. JW Tsao, D. Vernet, G. Nolazco, J. Artaza, **NF Gonzalez-Cadavid** Androgen modulation of cell lineage commitment by muscle-derived stem cells (MDSC).. Presented at the Tenth Research Centers in Minority Institutions (RCMI) International Symposium on Health Disparities. Dec. 13-16. 2006. Puerto Rico.

C168. Kovanecz I, Ferrini MG, Davila HH, Rajfer J, **Gonzalez-Cadavid NF** Pioglitazone ameliorates penile corporalveno-occlusive dysfunction (CVOD) in a rat model of type 2 diabetes. Annual Meeting of AUA 2005 San Antonio, TX, USA J Urol 2005; 173(4S): 283-4.

C169. Davila HH, Miranda-Sousa AJ, Kovanecz I, Carrion RE, Miller C, **Gonzalez-Cadavid NF**. Effect of bilateral cavernosal nerve resection on the histological alterations in the penile vascular system. Annual Meeting of AUA 2005 San Antonio, TX, USA. J Urol 2005; 173(4S): 288.

C169. Ferrini MG, Kovanecz I, Nolazco G, Vernet D, Rajfer J, **Gonzalez-Cadavid NF**. Reduction of the fibrotic plaque in a rat model of Peyronie's disease by a long term PDE5 inhibition. Annual Meeting of AUA 2006 Atlanta, GA, USA J Urol 2006; 175(4S): 320-21.

C170. Kovanecz I, Ferrini MG, Nolazco G, Rajfer J, **Gonzalez-Cadavid NF**. Aging related corpora veno-occlusive dysfunction in rat is ameliorated by pioglitazone. Annual Meeting of

AUA 2006 Atlanta, GA, USA J Urol 2006; 175(4S): 326-27.

C171. Kovanecz I, Rambhatla A, Ferrini MG, Sanchez S, Rajfer J, **Gonzalez-Cadavid NF**.Tadalafil prevents corporal veno-occlusive dysfunction (CVOD) that occurs following bilateral cavernosal nerve resection in the rat. Annual Meeting of SMSNA 2006, Las Vegas, NV, USA.

C172. Kovanecz I, Rambhatla A, Ferrini MG, Vernet D, Sanchez S, Rajfer J, **Gonzalez-Cadavid NF**.Amelioration of cavernosal nerve resection induced corporal veno-occlusive dysfunction (CVOD) by long term sildenafil treatment in rats. Annual Meeting of SMSNA 2006, Las Vegas, NV, USA

C173. J. N. Artaza. R. Singh **N Gonzalez-Cadavid** Myostatin Effects on C3H 10T (1/2) mesenchymal multipotent cells through the Smad signaling pathway: a microarray approach. Presented at the Western American Federation for Medical Research, February, 1-4, 2006. Sunset Center Carmel, CA, USA. Winner of the Endocrine Scholar Award

C174. J N Artaza. R. Singh, **N Gonzalez-Cadavid** Myostatin Promotes Collagen Deposition in C3H 10T (1/2) mesenchymal multipotent cells through the Smad signaling pathway. Presented at the ENDO-'2006. The Endocrine Society's 88ndAnnual Meeting June,24-27 2006. Boston. MA.

C175. Jorge N. Artaza, Suzanne Reisz-Porszasz, Joan S Dow, Robert A. Kloner, Shalender Bhasin, and **Nestor F. Gonzalez-Cadavid**. Myostatin overexpression reduces heart mass in the mouse but does not affect cardiac function. Presented at the Tenth Research Centers in Minority Institutions (RCMI) International Symposium on Health Disparities. Dec. 13-16. 2006. Puerto Rico

C176. Jorge. N. Artaza, Rajan Singh, Monica Ferrini, Melissa Braga, James Tsao and **Nestor. Gonzalez-Cadavid** Myostatin Promotes the Expression of Pro-fibrotic Factors and Collagen Deposition through the Smad Signaling Pathway.. Presented at the Tenth Research Centers in Minority Institutions (RCMI) International Symposium on Health Disparities. Dec. 13-16. 2006. Puerto Rico.

C177. Rajan Singh, Melissa Braga, Jorge N. Artaza, Shalender Bhasin and **Nestor F Gonzalez-Cadavid.**Testosterone Modulates Transforming Growth factor- β Signaling Pathway Genes in Mesenchymal Pluripotent C3H 10T1/2 Stem Cells: implications in regulation of body composition" Presented at the Western American Federation for Medical Research, February, 1-4, 2006. Sunset Center Carmel, CA, USA..

C178. Thomas R. Magee, Jorge N. Artaza, Monica G. Ferrini, Dolores Vernet, Freddi I. Zuniga, Liliana Cantini, Suzanne Reisz-Porszasz, Jacob Rajfer and **Nestor F. Gonz alez-Cadavid** Myostatin short interfering hairpin RNA gene transfer increases muscle mass and reduces interstitial connective tissue".. Presented at the Western American Federation for Medical Research, February, 1-4, 2006. Sunset Center Carmel, CA, USA.

C179. Singh Rajan, Taylor WE, Jasuja R, Salehian B, Braga M, **Gonzalez-Cadavid NF**.

Testosterone inhibition of insulin signaling pathway genes and adipogenic genes observed in 3T3-L1 mouse preadipocyte cells by expression profile. Journal of Investigative Medicine 54 (1): S151-S151 419 Suppl. S, Jan 2006.

C180. Rajan Singh, Melissa Braga, Jorge N. Artaza, **Nestor F Gonzalez-Cadavid**, Jibong Lei and Shalender Bhasin. Testosterone Effects on Myogenic Differentiation in C3H 10T1/2 Cells are mediated through Cross-Talk Between Androgen Receptor / β-catenin / TCF4 and Transforming growth factor- β Signaling Pathways: Possible intermediate role of follistatin" Presented at the ENDO-'2006. The Endocrine Society's 88[nd]Annual Meeting June,24-27 2006. Boston. MA.

C181. Singh R, Braga M, Artaza J, Jasuja R, Taylor W, Miki R, Gupta, V, Satyesh K Sinha, **Gonzalez-Cadavid N**, Bhasin S, Rajavashisth T B. Testosterone-Mediated Increase in Myogenesis in vitro and in vivo: Intermediate Role of Follistatin. Tenth Research Centers in Minority Institutions (RCMI) International Symposium on Health Disparities. Dec. 13-16. 2006. Puerto Rico.

C182. Jorge N. Artaza, Suzanne Reisz-Porszasz, Joan S Dow, Robert A. Kloner, Shalender Bhasin, and **Nestor F. Gonzalez-Cadavid**. Myostatin overexpression reduces heart mass in the mouse but does not affect cardiac function. Western American Federation for Medical Research, January 31-February 3, 2007. Sunset Center Carmel, CA, Journal of Investigative Medicine, Vol 55, Issue 01, January 2007. Abstract # 62

C183. TR Magee, Kovanecz I, LP Cantini, MG Ferrini, D Vernet, Hugo H Davila. J Rajfer, **NF Gonzalez-Cadavid** A short hairpin RNA targeting PIN (Protein Inhibitor of NOS) is more efficient than the anti-sense PIN RNA for gene therapy of aging-related erectile dysfunction in the rat. . American Urological Association May 19-24, 2007. Anaheim, CA.

C184. Ferrini MG, Kovanecz I, Sanchez S, Rajfer J, **Gonzalez-Cadavid NF**. Time course of corporal veno-occlusive dysfunction (CVOD) development and inducible nitric oxide synthase (iNOS) expression in rat penile tissue after cavernosal nerve damage. J Urol 177 (4): 262-62 Suppl. S, 2007
2007

C185. Ferrini MG, Cantini LP, Vernet D, Magee TR, Qian A, Gelfand RA, Rajfer J, **Gonzalez-Cadavid NF** Pro-fibrotic role of myostatin in Peyronie's disease.   American Urological Association May 17-22, 2008, Orlando, FL

C186. Ho MH, Heydarkhan S, Vernet D, Kovanecz I, Ferrini MG, Bathia NN, **Gonzalez-Cadavid NF**. Skeletal muscle-derived stem cells (MDSC) seeded on small intestinal submucosal (SIS) scaffolds stimulate vaginal repair in the rat. American Urological Association May 17-22, 2008, Orlando, FL

C187. Kovanecz I, Ferrini MG, Heydarkhan S, Nolazco G, Vernet D, Rajfer J, **Gonzalez-Cadavid NF.** Type 2 diabetes causes fibrosis in the media of the penile dorsal artery (PDA) and the aorta. American Urological Association May 17-22, 2008, Orlando, FL

C188. Nolazco G, Kovanecz I, Vernet D, Ferrini MG, Gelfand R, Tsao J, Magee T, Rajfer J, **Gonzalez-Cadavid NF**. Effect of muscle derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. American Urological Association May 17-22, 2008, Orlando, FL

C189**.** Toblli Jorge; Ferrini Monica G, Cao Gabriel, Vernet Dolores, Angerosa Margarita **Gonzalez Cadavid Nestor F** . Pioglitazone reduces renal fibrosis in a rat model of type 2 diabetes. Natl Kidney Found, Texas, April 2008

C190. **Gonzalez-Cadavid NF**. Molecular basis of Peyronie's disease. Invited speaker. Ann Meet SMSNA, Orlando, Fl, May 2008

C191. Ferrini MG, Davila H, Rajfer J, **Gonzalez-Cadavid NF** (2008) Chronic heavy smoking exacerbates erectile dysfunction and fibrosis of the penile corpora cavernosa and arterial media smooth muscle in rat models of type 1 diabetes and aging. Sex Med Soc North Amer (SMSNA) meeting, New York, NY.

C192. Toblli JE; Ferrini MG, Cao G, Vernet D, Angerosa M, **Gonzalez Cadavid NF** (2008). Pioglitazone moderates oxidative stress, apoptosis and the inflammatory response in the kidney of a rat model of type 2 diabetes mellitus. Am Soc Nephrol (ASN) Renal Week Meeting, Philadelphia (PA).

C193. Nolazco G, Toblli J, Kovanecz I, Gelfand R, Lue Y-H, **Gonzalez-Cadavid NF** (2009) Activation of the Oct-4 gene identifies stem cells in the kidney that are reduced by type 2 diabetes mellitus in a process counteracted by a PPARγ ligand independently from glycemic control. Endocrine Soc Meet, Washington DC.

C194. Vernet D, Heydarkhan S, Kovanecz I, Lue Y-H, Rajfer J, **Gonzalez-Cadavid NF** (2009). Characterization of endogenous stem cells from the mouse penis that express an embryonic stem cell gene and undergo differentiation into several cell lineages. Am Urol Assoc Meet, Chicago, IL, J Urol,

C195. Kovanecz I, Nolazco G, Ferrini MG, Rivera S, Vernet D, Rajfer J, **Gonzalez-Cadavid NF** (2009) Severe hyperglycemia, renal damage, and low serum testosterone are insufficient in the absence of obesity to cause corporal veno-occlusive dysfunction (CVOD) in a rat model of type 2 diabetes. Am Urol Assoc Meet, Chicago, IL, J Urol,

C196. Ferrini MG, Moon J, Rivera S, Rajfer J, **Gonzalez-Cadavid NF** (2009) Genetic Blockade of inducible nitric oxide synthase (iNOS) Intensifies the Development of Fibrosis in a Mouse Model of Diabetes Type 1 Am Urol Assoc Meet, Chicago, IL, J Urol,

C197. Ferrini MG, Moon J, Rivera S, Vernet D, Rajfer J, **Gonzalez-Cadavid NF** (2009) Antifibrotic and antioxidant therapies prevent the development of fibrosis in the penile corpora cavernosa associated with type 1 diabetes in the iNOS knock out mouse. Am Urol Assoc Meet, Chicago, IL, J Urol

C198. **Gonzalez-Cadavid NF**, Tsao J, Vernet D, Gelfand R, Nolazco G (2009) Modulation of cell lineage commitment by skeletal muscle derived stem cells, MDSC, from mdx and myostatin knockout mice. Military Health Research Forum 2009, Kansas City,

2010/11 to be inserted (6 abstracts)